National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395 rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

## NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.



## AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.



Canada

# Molecular Cloning and Characterization of Flavonol Sulfotransferase and Nucleoside Diphosphate Kinase cDNA Clones from *Flaveria bidentis*, and the Regulation of Flavonol 3-sulfotransferase in Cell Suspension Cultures

Sirinart Ananvoranich

A Thesis
in
The Special Individualized
Programme

Presented in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy at
Concordia University
Montreal, Quebec, Canada

September 1994

ISBN 0-612-01269-7

Canada

**CONCORDIA UNIVERSITY**

**School of Graduate Studies**

This is to certify that thesis prepared

By: **Ms. Sirinart Ananvoranich**

Entitled: **Molecular Cloning and Characterization of Flavonol Sulfotransferase and Nucleoside Diphosphate Kinase cDNA Clones from *Flaveria bidentis*, and the Regulation of Flavonol 3-Sulfotransferase in Cell Suspension Cultures**

and submitted in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy**

complies with the regulations of this University and meets the accepted standard with respect to originality and quality.

Signed by the final examining committee:

_____ Chair

_____ External Examiner

_____ External Examiner

_____ External to Program

_____ Examiner

_____ Examiner

_____ Supervisor

Approved_____

Chair of Department or Graduate Programme Director

_____19__ _____

Dean of Faculty

## ABSTRACT

Molecular Cloning and Characterization of Flavonol Sulfotransferase and Nucleoside Diphosphate Kinase cDNA Clones from Flaveria bidentis, and the Regulation of Flavonol 3-sulfotransferase in Cell Suspension Cultures

Sirinart Ananvoranich, Ph.D
Concordia University, 1994

Two cDNA clones encoding flavonol 3-sulfotransferase (pBFST3) and a flavonol sulfotransferase-like protein (pBFSTX), as well as two cDNA clones coding for nucleoside diphosphate kinases (pNDPK-a and pNDPK-b) were isolated and characterized from Flaveria bidentis by immunoscreening of an expression library with a polyclonal antibody raised against F. chloraefolia flavonol 3-sulfotransferase (F3-ST) and by a polymerase chain reaction-mediated strategy. Flaveria spp. accumulate sulfated flavonols whose biosynthesis is catalysed by a number of position-specific flavonol sulfotransferases (F-STs). Although the accumulation of sulfated flavonols appears to be tissue-specific, developmentally regulated, and to vary among related species, little is known about the mechanism of regulation controlling their biosynthesis. Cell suspension cultures of F. bidentis were used to study the effects of nutrients, phytohormones, elicitors and flavonols

on growth and F3-ST enzyme activity. None of the nutrients or elicitors tested exhibited significant effects on F3-ST activity. The synthetic auxin, 2,4-D was shown to induce F3-ST enzyme activity and the F3-ST transcript accumulation in cell cultures of F. bidentis, whereas kinetin did not alter the level of F3-ST activity. Treatments with either quercetin 3-sulfate or quercetin 3,7,4'-trisulfate reduced F3-ST enzyme activity in cell cultures, but had no effect on the transcript levels. These results are discussed in relation to the putative role of flavonoid conjugates in the regulation of auxin transport. Moreover, the cross-reaction of pNDPK-a and pNDPK-b to the F3-ST antibody led to an investigation of a common immuno-epitope. A putative binding site for PAPS, cosubstrate of F-STs, is proposed, based on the high similarity region between NDPK-a and F3-ST, to be located at the amino acid residues 161-199 of the F3-ST amino acid sequence. In addition, members of the F-ST and NDPK gene families in F. bidentis were estimated by Southern hybridization using probes prepared from the inserts of pBFST3, pBFSTX and pNDPK-b cDNA clones. The sulfotransferase gene family apparently consists of three to five members; the nucleoside diphosphate kinase consists of at least two members.

## ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my supervisor, Prof. Ragai K. Ibrahim, for his generous assistance, guidance and support throughout the course of the investigation and preparation of this dissertation. I am also grateful to him for providing a financial support needed for completion of my study.

I would like to thank Dr. Patrick Gulick for his constructive advice and discussions throughout my study.

I would also like to thank Dr. Luc Varin for his valuable advice and for providing materials used in the study.

My deep appreciation is expressed to Dr. Antonin Gauthier, Dr. Johnny Basso and Dr. Wanchai De-Eknamkul for their friendship and encouragement.

I would also like to express my appreciation to my colleagues, Hubert Gagnon, Pierre Laflamme, Susan-Marie Aitken, Wei Shen, Dr. Jennifer Saleeba, Dr. Jacques Grandmaison, Dr. Sabine Gubatz, Dr. Sylvie Attucci, and Jacynthe Seguin for creating an enjoyable and unforgettable atmosphere.

Finally, I would like to thank the Canadian International Developing Agency for providing a financial support during my study.

# TABLE OF CONTENTS

Page

LIST OF TABLES.....xii

LIST OF FIGURES.....xiv

LIST OF ABBREVIATIONS.....xviii

**CHAPTER I**

A. Introduction .....1

B. Aim of Work .....3

B.1 Characterization of F. bidentis cell suspension cultures.....3

B.2 Molecular cloning and regulation of flavonol 3-sulfotransferase in cell suspension cultures .....3

B.3 Molecular cloning and characterization of flavonol sulfotransferases .....4

C. Review of Literature .....5

C.1 Significance of flavonoids .....5

C.2. Biosynthesis of flavonols .....6

C.3 Key enzymes in flavonol sulfate biosynthesis .....11

C.3.1 Phenylalanine ammonia-lyase (PAL) .....13

C.3.2 Chalcone synthase (CHS) .....18

C.3.3 Flavonol sulfotransferases (STs) .....23

D. Literature Cited .....32

**CHAPTER II**

**Effects of Nutrients, Phytohormones, Elicitors, and Flavonols on Growth and Flavonol Sulfotransferase Activity of *Flaveria bidentis* Cell Suspension Cultures**.....43

A. Abstract .....43

B. Introduction .....43

C. Materials and Methods .....46

C.1 Plant Material .....46

C.2 Chemicals .....47

C.3 Characterization of flavonol sulfotransferase activity of *F.bidentis* .....47

C.4 Growth measurement of *F. bidentis* cell culture ...48

C.5 Measurement of major nutrients .....49

C.6 Treatment of plant cell cultures .....50

C.7 Preparation of extracts for enzyme assay .....51

C.8 Flavonol sulfotransferase assay .....51

C.9 Western blot analysis .....52

D. Results .....52

D.1 Characterization of flavonol sulfotransferase of *F. bidentis* cell cultures .....52

D.2 Nutrient depletion by *F. bidentis* cell cultures ..53

D.3 Treatment of cell cultures with nutrients, phytohormones, elicitors, and flavonols .....54

E. Discussion .....55

F. Literature Cited .....58

**CHAPTER III**

**Cloning and Regulation of Flavonol 3-Sulfotransferase in Cell Suspension Cultures of *F. bidentis*** .....74

A. Abstract .....74

B. Introduction .....75

C. Materials and Methods .....77

C.1 Plant Material .....77

C.2 Chemicals .....77

C.3 cDNA cloning of F3-ST .....78

C.4 Treatment of plant cell cultures .....79

C.5 Preparation of protein extracts .....80

C.6 Sulfotransferase assay .....80

C.7 Protein determination .....81

C.8 Northern blot analysis .....81

C.9 Western blot analysis .....82

D. Results .....82

D.1 Characteization of F3-ST cDNA clone .....82

D.2 Sequence analysis of pBFST3 .....83

D.3 F3-ST activity during growth of *F. bidentis* cell cultures .....84

D.4 Effects of 2,4-D on F3-ST .....84

D.5 Effect of flavonols on F3-ST .....85

E. Discussion .....86

F. Literature Cited .....92

**CHAPTER IV**

**Molecular Cloning and Characterization of Flavonol Sulfotransferase-Like cDNA from F. bidentis** .....110

A. Abstract .....110

B. Introduction .....111

C. Materials and Methods .....112

C.1 Plant Material .....112

C.2 Chemicals .....112

C.3 cDNA library construction and pBluescript excision.....112

C.4 Genomic DNA isolation .....113

C.5 Cloning and expression strategy .....114

C.6 DNA sequencing .....115

C.7 Polymerase chain reaction .....117

C.8 Cloning of PCR fragments .....117

C.9 Colony hybridization .....118

C.10 Southern blot analysis .....119

C.11 Northern blot analysis .....120

C.12 Preparation of protein extracts from E. coli and yeast .....121

C.13 Sulfotransferase assay .....122

C.14 Western blot analysis .....123

D. Results .....123

D.1 Molecular cloning and characterization of a flavonol sulfotransferase-like cDNA clone .....123

D.2 Northern and Southern blot analysis .....126

E. Discussion .....127

F. Literature Cited .....132

**CHAPTER V**

**Molecular and Biochemical Characterization of Two Nucleoside Diphosphate Kinase cDNA Clones from F. bidentis** .....147

A. Abstract .....147

B. Introduction .....147

C. Materials and Methods .....150

C.1 Plant Material .....150

C.2 Chemicals .....150

C.3 F. bidentis cDNA library construction and immunoscreening .....150

C.4 Southern blot analysis .....151

C.5 Northern blot analysis .....152

C.6 DNA probe preparation .....152

C.7 DNA sequencing .....153

C.8 NDPK expression in E. coli .....153

C.9 Western blot analysis .....155

D. Results .....155

D.1 Characterization of nucleoside diphosphate kinase cDNA clones .....155

D.2 Sequence analysis of pNDPK-a and pNDPK-b .....156

D.3 Northern and Southern blot analysis .....157

E. Discussion .....158

F. Literature Cited .....161

**CHAPTER VI**

A. Discussion and Conclusions .....174

B. Perspective for future works .....180

C. Literature Cited.....183

# LIST OF TABLES

**CHAPTER I**

**Table 1.** Reported cDNA clones encoding phenylalanine ammonia-lyase (PAL) .....14

**Table 2.** Reported cDNA clones encoding chalcone synthase (CHS) .....19

**Table 3.** Reported cDNA clones encoding sulfotransferases (STs) .....29

**CHAPTER II**

**Table 1.** Treatments of F. bidentis tissue cultures using different conditions and compounds .....62

**Table 2.** Fresh weight, cell viability and flavonol sulfotransferase activity of F. bidentis cell cultures observed under different growth conditions ..63

**CHAPTER III**

**Table 1.** Substrate specificity of F3-ST cloned in E. coli .....94

**CHAPTER IV**

**Table 1.** Oligonucleotide primers used in cloning of pBFSTX .....134

**CHAPTER V**

**Table 1.** The phosphate-incorporating activity of pNDPK-a and pNDPK-b .....164

# LIST OF FIGURES

**CHAPTER I**

**Figure 1.** Different flavonoid classes .....8

**Figure 2.** Phenylpropanoid and flavonoid pathways .....9

**Figure 3.** Amino acid alignment of reported sulfotransferases.....30

**CHAPTER II**

**Figure 1.** Three-day-old F. bidentis cell suspension cultures grown in MS medium .....64

**Figure 2.** Partial purification of F-ST from F. bidentis tissue cultures. (A) Elution profile of the F-ST activities after chromatofocusing on Mono P. (B) Autoradiogram of the chromatographed enzyme reaction products from Mono P fractions. (C) Western blot analysis of the protein fractions ...65

**Figure 3.** Growth cycles of F. bidentis cell suspension cultures. (A) Depletion of major nutrients in MS medium. (B) Depletion of major nutrients in B5 medium. (C) Profiles of fresh weight and F3-ST activity of cultures grown in MS medium. (D) Profiles of fresh weight and F3-ST activity of cultures grown in B5 medium .....69

**CHAPTER III**

**Figure 1.** Expression of pBFST3 in E. coli (A) Western blot analysis of E. coli lysates of pBFST3. (B) Autoradiogram of the chromatographed enzyme reaction products from the E. coli lysate of pBFST3 .....95

**Figure 2.** The nucleotide and amino acid sequences of pBFST3.....97

**Figure 3.** The amino acid sequence alignment of F. bidentis pBFST3, F. chloraefolia pFST3 and pFST4' ....98

**Figure 4.** Flavonol 3-sulfotransferase activity in relation to age of F. bidentis cell cultures .....99

**Figure 5.** 2,4-D treatments (A) Dose-response curve. (B) Time-response curve. (C) Northern blot analysis using pBFST3 as a probe .....101

**Figure 6.** F3-ST induction kinetics in response to 2,4-D and actinomycin D treatments .....105

**Figure 7.** The effect of different flavonols (A) on F3-ST activity. (B) Northern blot analysis of cell cultures following flavonol treatments .....107

**CHAPTER IV**

**Figure 1.** Amino acid alignment of reported sulfotransferases.....135

**Figure 2.** Schematic representation of the PCR-mediated cloning strategy for pBFSTX .....137

**Figure 3.** The nucleotide and deduced amino acid sequence of the pBFSTX insert .....139

**Figure 4.** The amino acid alignment of a putative flavonol sulfotransferase of F. bidentis (pBFSTX) with F. bidentis F3-ST (pBFST3), and F. chloraefolia F3-ST (pFST3) and F4'-ST (pFST4') .....140

**Figure 5.** Western blot analysis of lysates of E. coli XL1-Blue expressed pBFSTX .....141

**Figure 6.** Northern blot analysis of poly(A)$^+$ RNA from F. bidentis terminal buds and cell cultures ....143

**Figure 7.** Southern blot analysis (A) probed with the cDNA inserts of pBFSTX. (B) probed with the cDNA inserts of pBFST3 .....145

**CHAPTER V**

**Figure 1.** The nucleotide and amino acid sequence of pNDPK-a and pNDPK-b .....165

**Figure 2.** Western blot analysis of E. coli lysates expressed pNDPK-a and pNDPK-b gene products .....166

**Figure 3.** Deduced amino acid sequence alignment of two cDNA clones of Flaveria bidentis NDPK-a and NDPK-b with the reported NDPK sequences .....168

**Figure 4.** The amino acid alignment of a high similarity region of F. bidentis NDPK and F3-ST .....170

**Figure 5.** Northern blot analysis of pNDPK-b .....171

**Figure 6.** Southern blot analysis of NDPK genes .....172

**CHAPTER VI**

**Figure 1.** A proposed model that sulfated flavonols may be involved in an interactive mechanism controlling auxin polar transport .....177

# CHAPTER I

## A. Introduction

Plants produce a variety of secondary metabolites, such as alkaloids, flavonoids, and terpenoids, to mention a few. These are believed to serve a variety of functions as diverse as deterrents of predators, and attractants for pollinators and symbionts (Mann, 1987). Secondary metabolites can be defined as organic compounds having no recognized role in the maintenance of fundamental life processes in the organisms that synthesize them (Bell, 1981). Flavonoids constitute one of the largest groups of naturally occurring phenolic compounds. Their chemistry and biochemistry are well documented (Harborne, 1988). The expression of many of the genes involved in their biosynthesis has been shown to be developmentally and environmentally regulated (van der Meer et al., 1993; Koes et al., 1994).

Flavonoids have generally been reported to play important roles as flower pigments, as stress metabolites that are formed in response to UV irradiation and microbial challenging, as inducers of pollen germination processes, and as regulators of auxin polar transport (for review see Koes et al., 1994). The diverse functions of flavonoids may result from their structural variations which encompass a number of flavonoids with different functional groups and substitutions (Heller and Forkmann, 1988). Flavonoids are categorised

depending upon their level of oxidation into subgroups including chalcones, flavanones, flavones, dihydroflavonols, flavonols, isoflavones and anthocyadinins. Each subgroup may undergo different substitutions, namely hydroxylation, methylation, glycosylation, prenylation, or sulfation. Flavonoid sulfation has been the most recently recognized modification (Harborne, 1975).

Flavonoid sulfates are of common occurrence in plants (Barron et al., 1988), especially in the Asteraceae. The most commonly found compounds are mono- and disulfate esters of flavones and flavonols or their methyl ethers and, less commonly, of their glycosylated derivatives. Tri- and tetrasulfated esters have also been identified, although these compounds have a more limited taxonomic distribution (Hannoufa et al., 1994). Recently, a number of position-specific flavonol sulfotransferases (F-STs) have been characterized from Flaveria chloraefolia (Varin and Ibrahim, 1989) and F. bidentis (Varin and Ibrahim, 1991). Different enzymes exhibited strict specificity for position 3 of flavonol aglycones (F3-ST), position 3' or 4' of flavonol 3-sulfate (F3'/F4'-ST) and position 7 of flavonol 3,3'- or 3,4'-disulfates (F7-ST), thus establishing an enzymatic sequence for the formation of flavonol polysulfates (Varin, 1992).

F. bidentis (Asteraceae) accumulates flavonol mono- to tetrasulfate esters (Barron et al., 1986) and exhibits several ST activities (Varin et al., 1987). F-ST activity of F.

bidentis has recently been shown to be regulated with respect to plant development, being highest in the shoot tips and the first pair of expanded leaves and lowest in mature leaves (Hannoufa et al., 1991). In contrast, cell suspension cultures of F. bidentis exhibit very low constitutive levels of F-ST activity and do not accumulate any flavonol sulfate esters (Bleichert et al., 1989).

## B. Aim of Work

### B.1 Characterization of F. bidentis cell suspension cultures

Cell suspension cultures of F. bidentis were used in this study as a model system to demonstrate the regulation of flavonol sulfotransferase activity. Different compounds affecting flavonol sulfotransferase activity were tested. These were chosen based on the previous reports indicating that these compounds affect the early biosynthetic enzymes of the flavonoid pathway. Compounds tested included the medium nutrients, phytohormones, biotic and abiotic elicitors and some flavonols.

### B.2 Molecular cloning and regulation of flavonol 3-sulfotransferase in cell suspension cultures of F. bidentis

The tested compounds which showed an effect on flavonol sulfotransferase activity were further investigated to demonstrate the level of regulation. In order to study the

regulation of F3-ST gene expression, a gene specific probe from F. bidentis was isolated. A cDNA clone encoding F3-ST was isolated and characterized from a F. bidentis cDNA expression library by immunoscreening using a polyclonal antibody raised against F. chloraefolia flavonol 3-sulfotransferase (Varin and Ibrahim, 1992).

**B.3 Molecular cloning and characterization of other flavonol sulfotransferases**

F. bidentis accumulates a variety of flavonol sulfate esters ranging from mono- to tetrasulfated flavonols, thus suggesting the presence of a number of genes encoding position-specific flavonol sulfotransferases. I am interested in finding other F-ST genes, in addition to the cDNA encoding F3-ST. Different strategies, including immunoscreening and polymerase chain reaction (PCR), were employed to obtain other F-ST clones. Genes encoding known substrate-specific flavonol sulfotransferases would be used for further studies of the pattern of gene regulation in relation to the presence of various sulfated flavonols.

In order to provide more details for each section of this study, the following chapters will be self-contained, each consisting of a specific Introduction, Materials and Methods, Results, Discussion and References sections. The last chapter of this dissertation will include a general discussion and

conclusions of the present study.

The following chapter will provide the basic information related to flavonol sulfate biosynthesis, including the significance of flavonoids in plants, biosynthetic pathways leading to the formation of flavonols and their conjugated forms, and a brief review of the studies on regulation of the key enzymes in flavonol polysulfate biosynthesis.

## C. Review of Literature

### C.1 Significance of flavonoids

Flavonoid compounds constitute one of the most abundant classes of plant secondary metabolites. Besides their roles as flower pigments, various flavonoids have been reported as stress metabolites that are formed in response to UV irradiation (Hahlbrock and Scheel, 1989; Chapple et al., 1992; Li et al., 1993) and microbial attack (Dixon et al., 1983; Lamb et al., 1989); as regulators of auxin polar transport (Jacobs and Rubery, 1988; Faulkner and Rubery, 1992); as stimulators of pollen germination (Pollak et al., 1993; Vogt et al., 1994) and as inducers of nodulation genes in Rhizobium-legume interaction (Long, 1989). In addition, they may play a role in the cohabitation of a host plant with the parasitic Striga species (Lynn and Chang, 1990).

Flavonoid conjugates (methylated, glycosylated, prenylated, and sulfated derivatives) have recently drawn much

interest in several studies involving the signalling and regulation in plant microbe-interaction (Firmin et al., 1986; Djordjevie et al., 1987; Kosslak et al., 1987; Redmond et al., 1986), male sterility (van der Meer et al., 1992) and auxin polar transport (Jacob and Rubery, 1988). Although its relation to phytoalexin synthesis is not yet clear, the isoflavone *O*-methyltransferase of alfalfa cell cultures has recently been reported to be induced ca 200-fold upon treatment with Colletotrichum lindemuthianum cell wall extract (Edwards and Dixon, 1991). Both glucosylation and deglucosylation of the flavonol, kaempferol have been proposed to control the level of pollen germination inducers (Pollak et al., 1993). In addition, prenylation of isoflavones has been reported to confer resistance to fungal attack (Dixon et al., 1983), and flavonol sulfation has been proposed to be involved in growth and development of Flaveria species (Hannoufa et al., 1991).

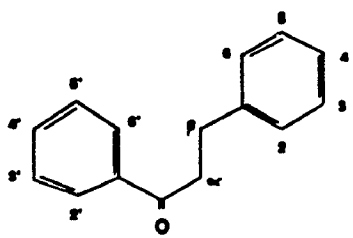
**C.2 Biosynthesis of flavonols**

Flavonols constitute a major subclass of flavonoid secondary metabolites. Their basic structure consists of two aromatic rings joined together by a three carbon unit ($C_6$-$C_3$-$C_6$). The two aromatic rings are derived from different pathways; ring A is formed from three acetate units via polyketide synthesis, whereas rings B and C are derived from a phenylpropane residue originating from phenylalanine via the

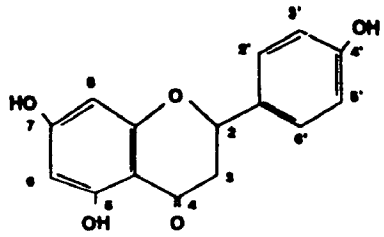
shikimate pathway. Depending upon the oxidation level of ring C, flavonoids are classified into different subclasses (Fig. 1). With a C=C-OH functional group of ring C (the $C_3$ unit), these compounds are designated as flavonols (for review see Harborne, 1988).

The basic structure of flavonols is derived from two biosynthetic pathways: the phenylpropanoid and the flavonoid pathways (Fig. 2). The phenylpropanoid pathway begins with *L*-phenylalanine which is irreversibly diverted from primary to secondary metabolism. Phenylalanine ammonia-lyase (PAL, a) catalyses the first committed step in this pathway. It catalyses the deamination of *L*-phenylalanine giving rise to *trans*-cinnamic acid. *Trans*-cinnamic acid is further hydroxylated by cinnamate 4-hydroxylase (C4H, b) to yield hydroxycinnamate (4-coumaric acid). The latter is activated by hydroxycinnamate CoA ligase (c) to give 4-coumaroyl CoA (Heller and Forkmann, 1988).

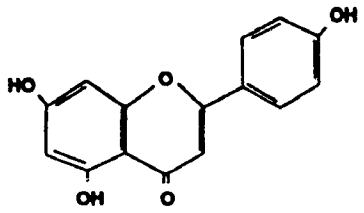
Flavonoid biosynthesis starts with the stepwise condensation of one molecule of 4-coumaroyl CoA with three units of malonyl CoA. This first committed step of the flavonoid pathway is catalysed by chalcone synthase (CHS, d). The central $C_{15}$ intermediate formed in this pathway, naringenin chalcone (4,2',4',6'-tetrahydroxychalcone), is stereospecifically transformed to the flavonone, 2*S*-naringenin by chalcone isomerase (CHI, e). The latter undergoes further steps of oxidation resulting in the formation of the various



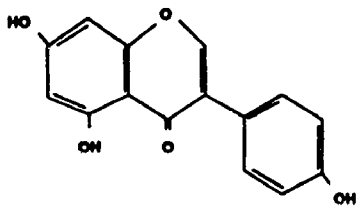
Chalcone



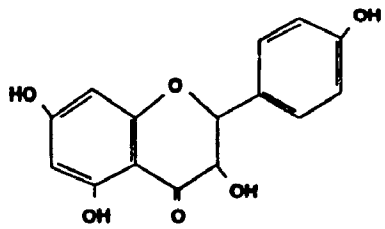
Flavanone



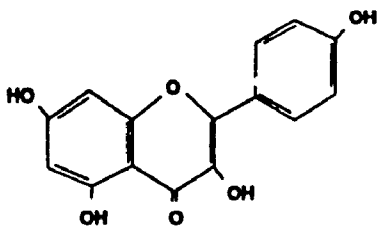
Flavone



Isoflavone



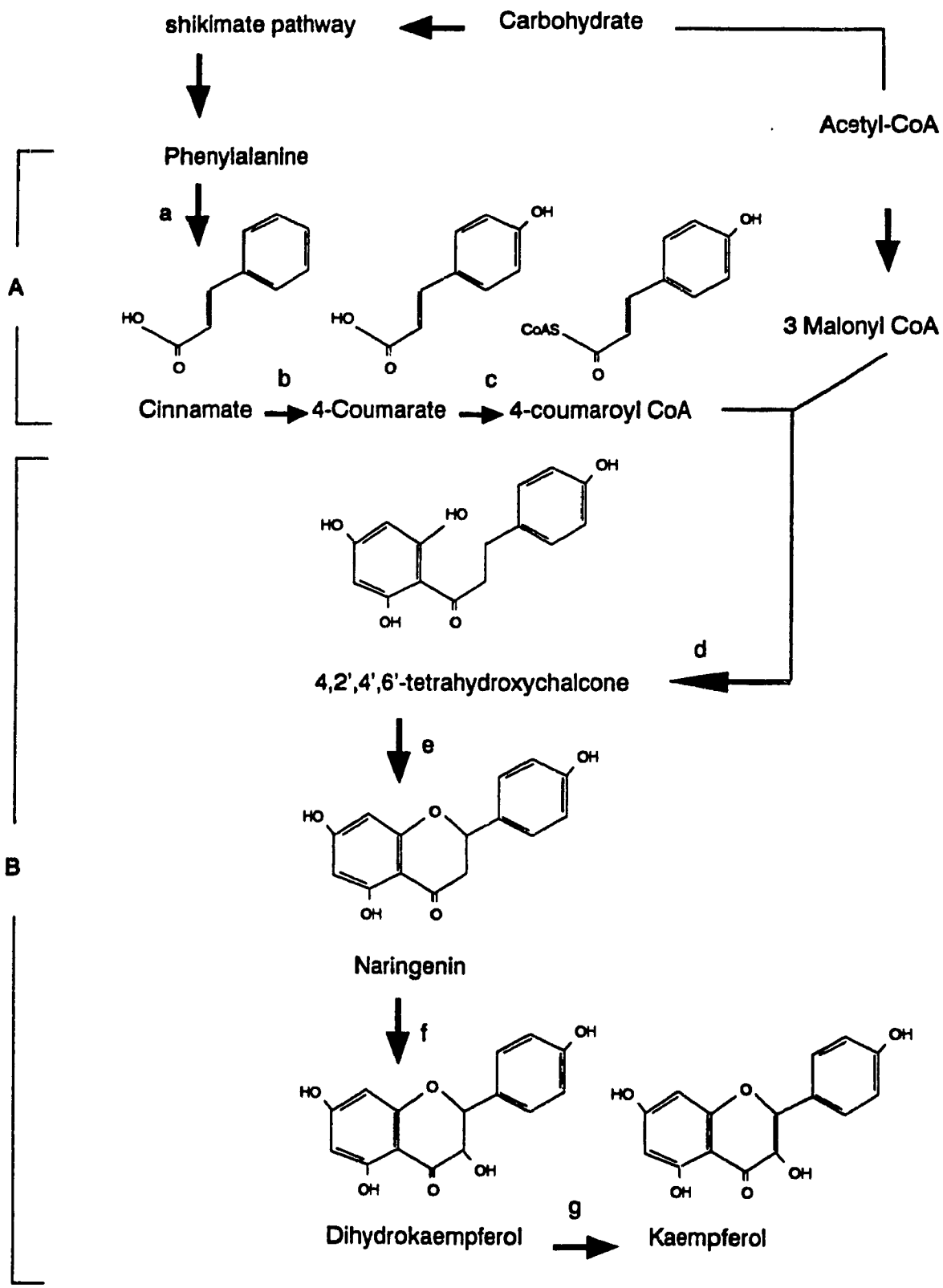
Dihydroflavonol



Flavonol

**Figure 1.** Different flavonoid classes.

**Figure 2.** Phenylpropanoid (A) and flavonoid (B) pathways, adapted from Heller and Forkmann, 1988. Biosynthetic enzyme **a** is phenylalanine ammonia-lysase; **b**, cinnamate 4-hydroxylase; **c**, hydroxycinnamate CoA ligase; **d**, chalcone synthase; **e**, chalcone isomerase; **f**, flavonone 3-hydroxylase and **g**, flavonol synthase.


shikimate pathway
Carbohydrate
Acetyl-CoA
Phenylalanine
a
A
3 Malonyl CoA
b
c
Cinnamate
4-Coumarate
4-coumaroyl CoA
d
4,2',4',6'-tetrahydroxychalcone
e
B
Naringenin
f
Dihydrokaempferol
g
Kaempferol

flavonoid classes, including isoflavones, flavones, flavonols and anthocyanidins.

Flavonols are formed from the flavanones which require dihydroflavonols as biosynthetic intermediates. The latter are formed by the hydroxylation of flavanones at position 3, catalysed by flavanone 3-hydroxylase (f). A double bond between C-2 and C-3 of dihydroflavonols is then introduced by flavonol synthase (g) resulting in flavonols.

Flavonols are further modified by various conjugation reactions, namely *O*-methylation, *O*-glycosylation, prenylation or sulfation (reviewed by Ibrahim and Varin, 1993). Most, if not all, of these modification reactions are catalysed by substrate-specific and position-specific enzymes. Although each modification reaction requires different co-substrates and co-factors, the modified positions are frequently found at the 3- and 7-positions of ring A as well as 3'-, 4'- and/or 5'-positions of ring B of flavonols (Heller and Forkmann, 1988). Other positions of substitution are less common, for example, the glucosylation of positions 2'- and 5'- of partially *O*-methylated flavonols (Latchinian et al., 1987), the methylation of the position 5 of isoflavones (Khouri et al., 1988), and 6 of flavonols (Ibrahim et al., 1987), to mention a few.

### C.3 Key enzymes of flavonol sulfate biosynthesis

The pattern of flavonoid accumulation has been well

documented and shown to be accompanied by changes of the steady state levels of mRNAs encoding the enzymes involved, especially phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), the key enzymes in the phenylpropanoid and flavonoid pathways, respectively (Lawton et al., 1983; Dixon et al., 1983; Hahlbrock and Scheel, 1989; Lamb et al., 1989). The proposed functional roles of flavonoids are consequently assigned according to the sites or the levels of product accumulation, as well as the levels of gene expression or the activities of biosynthetic enzymes. In addition, some functional roles of flavonoids have been confirmed by the characterization of mutants deficient in flavonoid metabolites (see below).

A significant number of the genes involved in phenylpropanoid and flavonoid pathways have recently been cloned and characterized (for review, see van der Meer et al., 1993; Koes et al., 1994). These genes were cloned either by differential- and antibody screening of cDNA libraries, or by using transposable elements. At least eight different enzymes and five types of regulatory genes have been isolated and characterized.

PAL and CHS, the first enzymes in the phenylpropanoid and flavonoid biosynthetic pathways, respectively have been the most studied. The regulation of PAL and CHS gene expression are well documented (for review, see van der Meer et al., 1994) and give a framework for the studies of other genes

involved in the pathway. Flavonol sulfotransferases which are later enzymes in the biosynthesis of flavonol sulfates will then be described in relation to other reported sulfotransferases of plants and animals, and their possible functions will be discussed.

### C.3.1. Phenylalanine ammonia lyase (PAL)

PAL, the first enzyme in the phenylpropanoid pathway, diverts *L*-phenylalanine from primary metabolism into a variety of phenolic secondary metabolites, including hydroxycinnamic acids, coumarins, and lignins. The properties of PAL from various sources have been reviewed by Hanson and Havir (1981). PAL enzyme activity has been observed to change in response to various internal factors (spatial, temporal and developmental controls), and in response to external factors, such as light, fungal infection, wounding, applied growth regulators and pathway intermediates (for review see Hanson and Havir, 1981; Hahlbrock and Scheel, 1989).

Genes encoding PAL have been characterized from a number of plant species and reported to be members of multigene families in several plants (Cramer et al., 1989; Lois et al., 1989; Ohl et al., 1990; Gowri et al., 1991; Joos and Halbrock, 1992). A selection of reported genes encoding PAL is listed in Table 1. PAL genes from different systems exhibit a particular advantage for studies of the regulation of gene expression. Bean (Phaseolus vulgaris), parsley (Petroselinum

**Table. 1** Reported cDNA clones encoding phenylalanine ammonia-lyase (PAL)

| Organism | Referred to as | Coding sequence* (bp) | Reference |
|---|---|---|---|
| *Arabidopsis thaliana* | PAL-1, 2, 3 | 2178, 2154, 2085 | Wanner *et al.*, 1994 |
| *Glycine max* | PAL1 | 2142 | Frank and Vodkin, 1991 |
| *Ipomoea batatas* | PALO2 | 2124 | Tanaka *et al.*, 1989 |
| *Lycopersicon esculentum* | Pal | 2115 | Bloksberg and Kado, 1991 |
| *Medicago sativa* | PAL | 2178 | Gowri *et al.*, 1991 |
| *Nicotiana tabacum* | PAL1 | 2139 | Nagai *et al.*, 1994 |
| *Petroselinum crispum* | PA1-1 | 1741 | Lois *et al.*, 1989 |
| *Pisum sativum* | PAL<br>PA11, 2 | 2172<br>2172, 2175 | Kawamata *et al.*, 1992<br>Yamada *et al.*, 1992 |
| *Phaseolus vulgaris* | PAL5 | 1518 | Edwards *et al.*, 1985 |
| *Populus trichocarpa* x *P. deltoides* | PALGA | 2147 | Subramaniam *et al.*, 1993 |
| *P. kitakamiensis* | Palg2a | 2049 | Osakabe *et al.*, 1994 |
| *Rhodosporidium toruloides* | PAL1<br>PAL2 | 2081<br>2171 | Anson *et al.*, 1987<br>Rasmussen and Orum, 1991 |
| *Rhodotorula rubra* | PAL1<br>PAL2 | 2142<br>2022 | Filpula *et al.*, 1988a<br>Filpula *et al.*, 1988b |
| *Trifolium subterraneum* | PA11 | 2178 | Howles *et al.*, 1994 |
| *Vitis vinifera* | VVPAL | 1251 | Sparvoli *et al.*, 1994 |

* Size in base pairs of the coding sequence encoding PAL gene product in length (bp).

crispum), alfalfa (Medicago sativa), Arabidopsis, and poplar (Populus spp.) are the most currently studied species.

In bean, PAL is encoded by a multigene family (Cramer et al., 1989). Two genes of PAL gene family have been reported to be activated by wounding the hypocotyl, but only one member is activated by elicitors (Cramer et al., 1989). In addition, Liang et al. (1989a) showed by RNase protection with gene specific probes, that three bean-PAL genes are differentially expressed during development and in response to different environmental cues. While all three genes are expressed at high levels in roots, only PAL1 and PAL2 are expressed in the shoot, and only PAL1 is expressed in the leaves. All three PAL-genes are induced by mechanical wounding of the hypocotyls, but fungal infection only activates PAL1 and PAL3. Illumination of etiolated hypocotyls activates PAL1 and PAL2, but not PAL3. Moreover, the expression of PAL2 was later found to be important for the generation of lignin polymer in the early stages of vascular development (Liang et al., 1989b). In addition to developmental and environmental regulation, bean-PAL gene expression has been demonstrated to be regulated by the reaction product, *trans*-cinnamic acid. The addition of *trans*-cinnamic acid to elicitor-treated bean suspension culture inhibits the appearance of transcripts encoding PAL (Mavandad et al., 1990). The reporter gene, ß-glucuronidase (GUS) was also used under the control of PAL2 promoter in order to define the cis-elements responsible for

the tissue-specific expression (Leyva et al., 1992). These findings suggest that PAL expression is a key step in the regulation of lignin biosynthesis.

Parsley-PAL gene family has been reported to be regulated by treatments with UV, fungal elicitors, or wounding. The analysis of a PAL-GUS gene fusion expression revealed the promoter regions and cis-elements responsible for the induction (Lois et al., 1989).

In alfalfa, PAL transcript accumulation was reported to be induced by yeast extract, in addition to the endogenous accumulation of PAL transcripts in roots, stems, and petioles during growth and development (Gowri et al., 1989). Moreover, endogenous levels of *trans*-cinnamic acid and *trans*-4-coumaric acid, the phenylpropanoid pathway intermediates, were observed to affect PAL expression, in a manner similar to that found in the bean system. However, the compartmentalized pools of *t*-cinnamic acid and *t*-4-coumaric acid, have not been detected (Orr et al., 1993).

Soybean-PAL gene expression was monitored during Rhizobium-soybean interaction. This symbiotic interaction is a species-specific mechanism leading to nodule development (Djordjevic et al., 1987). During nodule formation, PAL expression was found to be coordinately expressed with CHS. Only one specific PAL member of the three soybean nodule-PAL gene family is induced bu Rhizobium infection, but not by stress or pathogen interaction (Estabrook and Sengupta-

Gopalan, 1991).

Arabidopsis thaliana, a small crucifer, is a model plant offering a number of advantages for genetic and molecular biological studies (Meyerowitz, 1989). Phenylpropanoid biosynthesis genes have been studied in relation to temporal and spatial gene regulation during development. The regulation of PAL gene expression was examined using the functional properties of PAL promoter and an Arabidopsis transformation system. Arabidopsis-PAL is expressed in a tissue-specific manner, as well. The promoter is activated in early seedling development, and in vascular tissue of mature plants. The promoter is active in the root tips and shoot apical meristems. In the flower, its expression is found in the sepals, anthers and carpels, but not in petals (Ohl et al., 1990). A similar pattern of regulation was reported in relation to the appearance of anthocyanin pigments (Kubassek et al., 1992).

As to lignin biosynthesis, PAL cDNA clones obtained from poplar have been used to study the growth and development of woody plants. PAL mRNA accumulation in poplar cell suspension cultures was shown to be induced by treatment with biotic elicitors such as cell wall polysaccharides derived from Phytophthora megasperma and Fusarium oxysporum (Moniz de Sa et al., 1992). Moreover, PAL gene expression is regulated in a tissue- and organ-specific manner, as well as by developmental processes occurring in the leaves and shoots (Subramaniam et

al., 1993). In addition to the above studies, similar regulation studies of PAL expression have been reported from other plant systems, such as sweet potato (Tanaka et al., 1989), tomato (Bloksberg and Kado, 1991), pea (Kawamata et al., 1992; Yamada et al., 1992), potato (Joos and Hahlbrock, 1992), and grape (Sparvoli et al., 1994).

**C.3.2 Chalcone synthase (CHS)**

Chalcone synthase generates a variety of flavonoid intermediates. A selection of genes encoding CHS are listed in Table 2. Distinct patterns of gene regulation have been demonstrated in the CHS gene family, similar to those of PAL multigene families. Selective members of the gene families involved in both pathways are concomitantly expressed in response to particular biological environments. CHS gene regulation has been studied using two different approaches, in addition to observation of the changes in the steady state mRNA levels. One approach is the introduction of chimeric genes consisting of gene-specific promoters fused to reporter genes into transgenic plant systems; the other is the introduction of gene-specific antisense genes into transgenic systems. Both approaches allow the deduction of regulation from a transient and/or stable gene expression. Currently, some locations of both positive and negative cis-acting elements have been identified to be important for CHS gene expression (for review see Dangl et al., 1989; van der Meer et

**Table. 2** Reported cDNA clones encoding chalcone synthase (CHS)

| Organism | Referred to as | Coding sequence* (bp) | Reference |
|---|---|---|---|
| *Arabidopsis thaliana* | CHS3 | 1188 | Feinbaum and Ausubel, 1988 |
| *Glycine max* | Chs6 | 1167 | Akada *et al.*, 1993a |
| | Chs2 | 1167 | Akada *et al.*, 1993b |
| | Chs7 | 1170 | Akada *et al.*, 1993c |
| *Hordeum vulgare* | CHS | 1197 | Rohde *et al.*, 1991 |
| *Medicago sativa* | CHS4-2, CHS6-4, | 1111, 858 | McKhann and Hirsch, 1994 |
| | CHS12-1, CHS4-1 | 1169, 1170 | |
| | CHS1, 2, 4 | 1170, 1170, 996 | Junghans *et al.*, 1993 |
| *Petunia hybrida* | ChsG | 1182 | Koes *et al.*, 1989 |
| *Petroselinum hortense* | CHS | 1196 | Reimond *et al.*, 1983 |
| *Pisum sativum* | CHS-1A, CHS-1B | 1170, 1170 | Hellens, 1994 |
| | CHS1 | 1170 | An *et al.*, 1993 |
| | CHS3 | 1169 | Ichinose *et al.*, 1992 |
| *Phaseolus vulgaris* | CHS | 1170 | Ryder *et al.*, 1987 |
| *Sinapis alba* | CHSG | 1188 | Batschauer *et al.*, 1991 |
| *Trifolium subterraneum* | CHS1, CHS2 | 1167, 1170 | Arioli *et al.*, 1994 |
| *Vitis venifera* | CHS | 1181 | Sparvoli *et al.*, 1994 |
| *Vigna unguiculata* | CHS | 1166 | TT Vo *et al.*, 1993 |

* Size in base pairs of the coding sequence encoding CHS gene product in length (bp).

al., 1993).

In bean, a CHS promoter was reported to be active only in the root apical meristems and in petals, but also weakly active in other floral organs, mature leaves and stems (Schmid et al., 1990). When fused to chloramphenicol acetyltransferase (CAT), bean-CHS promoter was strongly induced by fungal elicitors and exogenously applied *trans* 4-cinnamic acid (Mavandad et al., 1990). Specific cis-acting elements have later been demonstrated to consist of H-box and G-box (Loake et al., 1991, 1992). These findings suggest that a gene regulatory mechanism may sense the levels of pathway intermediates and, therefore, control the rate of the biosynthetic enzymes.

In parsley, CHS promoter was differentially active according to spatial and temporal cues, as well. *Cis*-acting elements responsible for a light-dependent response were subsequently defined by footprinting (Schulze-Lefert et al., 1989). The light-regulated elements of the CHS promoter, designated as boxI, and boxII, are composed of a G-box (for review see van der Meer et al., 1993). It has later been demonstrated that the activity of G-box *cis*-elements and G-box binding factor (GBF) is modulated by both light and the levels of phosphorylation in vivo and in vitro (Harter et al., 1994). A mechanism has recently been proposed by which *cis*-elements functioning in photocontrol are involved with the cyclic GMP and calcium mediated systems (Bowler et al., 1994). Potential

molecular mechanisms controlling *cis*-acting elements and DNA-binding protein activity are currently being investigated (Foster et al., 1994).

More recently, the finding that Arabidopsis-CHS mutants are sensitive to UV-B radiation, strongly suggests its important role in plant UV-B protection (Li et al., 1992). A similar response has also been reported in pea (Harker et al., 1990) and grape (Sparvoli et al., 1994).

In addition to the temporal, spatial and developmental regulation of CHS, two other avenues are currently being investigated. One area relates to stress-response, and the other to flower morphogenesis and pigmentation. In the case of stress response, a large body of evidence has been obtained from studies of plant-microbe interactions; whereas the information on flower morphogenesis and flavonoid gene expression has been derived from mutants deficient in CHS-gene expression (see below).

Flavonoid compounds have been reported to act as signal molecules in various Rhizobium-legume symbioses (Dixon, 1983). Legumes, such as alfalfa and soybean, are the most currently studied species. In addition to producing flavonoids as stress metabolites, legumes produce isoflavonoid signalling molecules in response to specific Rhizobium interactions. Specific members of CHS genes are expressed during Rhizobium-legume postinfection stage, and during nitrogen stress in nodule formation (Estabrook and Sengupta-Gopalan, 1991). The

induction kinetics are different for each member depending upon the conditions of exposure (Junghans et al., 1993; McKhann and Hirsch, 1994).

In petunia, defective CHS expression created by the introduction of an antisense CHS cDNA, driven by either the CAMV 35S promoter or the CHS promoter, results in a severe reduction in flower pigmentation (van der Krol et al., 1990). Transgenic plants containing the CHS antisense gene show defective gametogenesis (van der Meer et al., 1992). The transgenic plants having white anthers are male sterile due to the arrest in male gametophyte development, indicating an essential role of flavonoids in flower development. The mutants could be biochemically complemented by exposure to the wild-type stigma extract. The active constituent in that extract was later identified as kaempferol (Mo et al., 1992; Pollak et al., 1993). A similar function for flavonols, as inducers of pollen tube germination, was demonstrated in tobacco (Ylstra et al., 1992) where flavonol glucosides have been proposed to be the endogenous supply of the kaempferol pollen inducer. A more recent report indicates that the wound-induced kaempferol also gives rise to the induction of pollen tube germination, and the stigma is likely to be the sole source of flavonol (Vogt et al., 1994).

In addition to male sterility, flower pigmentation, the most studied area, has currently been investigated in order to elucidate the mechanism by which regulatory genes control a

distinct set of anthocyanidin target genes (Cone et al., 1993a, b). Petunia mutants have been shown to be transiently complemented by maize regulatory genes. This complementation, which utilised a heterologous expression of regulatory genes, suggested that the expression of target genes is determined by the promoter region of structural genes (Quattrocchio et al., 1993).

### C.3.3 Flavonol sulfotransferase (F-ST)

Enzymatic sulfations are catalysed by a heterogenous family of sulfotransferases (STs) which may have different, but often overlapping, substrate specificities especially in animal tissues (Wienshilboum and Otterness, 1994). STs transfer the sulfate moiety of 3'-phosphoadenosine 5'phosphosulfate (PAPS) to the hydroxyl or amine functional groups of acceptor molecules to form their corresponding sulfate esters. The major role of STs is the conversion of substrates into more hydrophilic conjugated forms. Thus, by altering their biological properties, STs might play a major role in regulating the intracellular levels of active or inactive metabolites.

In contrast with mammalian systems, enzymatic sulfation in plants is much less documented, and a fewer number of STs have been reported. One of the foremost studied ST groups is the flavonol ST (F-ST) family of Flaveria spp. The enzymatic synthesis of sulfated flavonols of Flaveria spp was first

reported by Varin et al. (1986, 1987). Subsequently, their biosynthetic enzymes, namely flavonol 3-, 3'-, 4'-, and 7-STs, were purified from F. chloraefolia and F bidentis by Varin and Ibrahim (1989, 1991, 1992). More recently, two cDNA clones encoding flavonol 3- and 4'-ST of F. chloraefolia have been reported (Varin et al, 1992). Concerning the biochemical reports other than the F-STs, two STs were reported from Lepidium sativum (Glendening and Poulton, 1988) and from Brassica juncea (Jain et al., 1990). Both ST enzymes were shown to be involved in the biosynthesis of glucosinolates.

Although the physiological significance of sulfated flavonoids in plants is still unclear, several reports have shed some light on their possible functions. Harborne (1975) suggested that sulfated flavonols may serve as a reservoir for sulfate ions. More recently, Faulkner and Rubery (1992) have showed that flavonols, conjugated either by sulfation or glucosylation, affect auxin efflux in microsomal preparations of Curcubita pepo. Moreover, the F3-ST which catalyses the first step in the biosynthesis of flavonol polysulfates, has been reported to be spatially and developmentally regulated in F. bidentis (Hannoufa et al., 1991). Enzymatic sulfation of flavonols might thus be involved in the regulation of developmental processes. The recent report of a sulfated product, 4-O-(6-O-sulfo-ß-D-glucopyranosyl)gallic acid (turgorin), as a putative regulator of leaf movement in Mimosa

pudica (Schildknecht et al., 1990) suggests that enzymatic sulfation might regulate the levels of active and inactive forms of the metabolite involved. However, the mechanism underlying the involvement of sulfated turgorin in leaf movement has yet to be identified. A ST-like protein has recently been localized in the pulvinus leaf tissue of Mimosa by Chamberland et al. (1994), and its cDNA encoding the protein has been recently obtained and will be used as a probe for studies of Mimosa leaf movement (L. Varin, personal communication). In addition, a sulfated glucosamine oligosaccharide, synthesized by a symbiotic *Rhizobium*, was reported as a specific signal for the interaction between host plants and their symbiotic organisms (Lerouge et al., 1990; Fisher and Long, 1992). The sulfated product would thus act as a signalling molecule, in addition to its possible roles in developmental processes.

In contrast with plant F-STs, a larger number of enzymes have been described from animal tissues. The enzymatic sulfation in animal systems is an important reaction in the biotransformation of a wide range of endogenous compounds, such as monoamine- and catecholamine neurotransmitters, steroid hormones, heparin and bile acids, as well as exogenous compounds such as drugs and xenobiotics (Roy, 1981). Sulfate conjugation is often regarded as a detoxification reaction. For example, the sulfation of liver bile acids decreases their re-absorption from the small intestine, and protects the liver

tissue from toxic detergent effects of several bile acids (Comer et al., 1993). The sulfation of steroids inactivates reactions and leads to the excretion of steroid sulfates, a process that would primarily occur in the liver (Driscoll et al., 1993). Although the physiological roles of sulfated steroids in other tissues have not yet been well defined, some conjugated steroids have been reported to act in certain target tissues. For instance, in uterus and breast tissues, enzymatic sulfation has been reported to regulate the level of biologically active free hormones (Driscoll et al., 1993; Bernier et al., 1994). In the brain, sulfated pregnenolone and dehydroepiandrosterone have been shown to be potent antagonists of the γ-aminobutyric acid ($GABA_A$) receptor and are capable of acting as neuroexcitory steroids (Paul et al., 1992).

Moreover, sulfated products such as glycoproteins and glycolipids (Sakac et al., 1992; Kabayashi et al., 1993), protoheparan sulfate and N-heparan sulfate (Shieh and Spear, 1994; Brandan and Hirschberg, 1988; Miller and Waechter, 1988), protein and/or glycoprotein (Leitinger et al., 1994), arylhydroxylamic acid, hydroxyarylamine, and benzyl alcohol (van der Goorbergh et al., 1985) have drawn attention to various enzymatic sulfations involved in cellular development in relation to cancer. For example, the sulfation of glycolipids is an important step in the biosynthesis of sulfogalactosylglycerolipid (SGG) for mammalian

spermatogenesis. The increase of SGG causes a premature release of spermatocytes, thus resulting in sterility (Sakac et al., 1992). In human renal carcinoma cells, the level of sulfolipids and sulfotransferase activity was found to markedly increase as compared to normal cells (Kobayashi et al., 1993). In addition, heparin and heparan sulfates have been implicated in the activity modulation of fibroblast growth factors (Ishihara et al., 1993), and in the regulation of herpes simplex virus-induced cell fusion (Shieh and Spear, 1994). Moreover, the sulfation of protein, either on the carbohydrate or tyrosine residues, is a post-translational step specific for the trans-golgi (Huttner, 1988; Leitinger et al., 1994). The sulfation of arylhydroxylamic acid, hydroxyarylamine and benzyl alcohol generates reactive intermediates which covalently bind to DNA, RNA and proteins in carcinogenic and toxicological events (van der Goorbergh et al., 1985).

The classification of STs has clearly been categorised, in addition to their various substrate-specificities, using amino acid sequence identities (Weinshilboum and Otterness, 1994). Depending on the degree of sequence identities, cytoplasmic STs are divided into three families: phenol-, hydroxysteroid-, and flavonol STs. Within a family, each individual ST amino acid sequence shows more than 40% identity to others. The phenol ST family consists of two subfamilies: phenol and estrogen STs, showing more than 60% amino acid

sequence identities among the STs within a subfamily. Since heparan sulfate ST and tyrosyl protein ST are membrane-bound enzymes and have distinct amino acid sequences from other cytoplasmic STs, they are categorised separately. Table 3 lists some characteristics of cDNA clones reported to encode STs. These include the mammalian phenol-, estrogen-, hydroxysteroid, tyrosyl proteins, and glucosaminoglycan N-acetylglucosamyl N-deacetylase/N (heparan) STs. In plants, only the F-STs have been well documented. To date, there are more than 20 cDNA sequences registered in GenBank that have been reported from animals, 3 cDNA sequences from plants, and 2 cDNA sequences from microorganisms.

Although extensive sequence homologies among cytoplasmic STs were observed within specific regions of the reported enzymes (Fig. 3), their functional relevance is, as yet, unknown. In theory, the STs may contain two substrate active sites, sharing a common site for the co-substrate, PAPS. The structure of PAPS which resembles the structure of ATP and ADP has led to a proposed region of a PAPS binding site. One proposed site was based on a conserved domain for the nucleotide binding site of ATP- and GTP-binding proteins (Hashimoto et al., 1992; Zhu et al., 1993). These proteins have a phosphate binding loop (P loop) which is a glycine-rich structure, GXXGXXXG, or $\alpha/\beta$ motif. This conserved domain is referred to as a classical mononucleotide binding domain (Hanks et al., 1988). The conserved regions among different

**Table. 3** Reported cDNA clones encoding sulfotransferases (STs)

| Gene product | Source | Organism | References |
|---|---|---|---|
| Phenol ST | liver | H. sapiens | Wilborn et al., 1993 |
| | liver | H. sapiens | Wood et al., 1994 |
| | liver | Mus musculus | Kong et al., 1993 |
| Estrogen ST | placenta | H. sapiens | Bernier et al., 1994 |
| | placenta | Bos taurus | Nash et al., 1988 |
| | liver | R. norvegicus | Demyan et al., 1992 |
| | adrocortical | Cavia cobaya | Oeda et al., 1992 |
| Aryl-ST | liver | Rattus norvegicus | Khan et al., 1993 |
| Aryl-ST I | brain | Homo sapiens | Zhu et al., 1993a |
| | liver | R. norvegicus | Ozawa et al., 1990 |
| II, III | brain | H. sapiens | Zhu et al., 1993b |
| IV | liver | R. norvegicus | Yerokun et al., 1992 |
| Hydroxysteroid ST | liver | R. norvegicus | Ogura et al., 1990a; 1990b |
| | liver | H. sapiens | Otterness et al., 1992 |
| | liver | H. sapiens | Comer et al., 1993 |
| | liver | H. sapiens | Kong et al., 1992 |
| Flavonol 3-ST | terminal buds | Flaveria chloraefolia | Varin et al., 1992 |
| 4-ST | terminal buds | F. chloraefolia | Varin et al., 1992 |
| 4-ST-like | leaf | Arabidopsis thaliana | Lacombe and Roby, 1993 |
| Hydroxylamine ST I, II | liver | R. norvegicus | Nagata et al., 1993 |
| Glucosaminyl-N-deceatylase | masatocytoma | Mus musculus | Eriksson et al., 1994 |
| N-ST | embryo | Caenorhabditis elegans | McCombie et al., 1993 |
| (or heparan ST) | MST cell culture | Mus musculus | Orellana et al., 1994 |
| | liver | R. norvegicus | Hashimoto et al., 1992 |

**Figure 3.** Amino acid alignment of reported sulfotransferases. The Flaveria flavonol sulfotransferases are pFST3 (Genbank accession number **M84135**), pFST4'(**M84136**). Mammalian hydroxysteroid, and oestrogen sulfotransferases are pHeST (**L20000**), pHhST (**843859**), pMST (**L02335**), pRST (**D14987**), pBeST (**M54942**), and pReST (**M86758**). Mammalian phenol- and arylsulfotransferases are pHaST-a (**L19955**), pHpST (**L19999**), pHaST-b (**L19956**), and pRmRT (**L19998**). Conserved amino acids in sulfotransferases are indicated in boxes. Gaps, introduced to obtain a maximum similarity are indicated by diamonds (♦). Identical amino acids are shown by dashes (-), and stop codons are shown by asterisk (*).

```
pFST3    (1-99)                MEDIIKTLPQ PTCSFLKHRF TLYKYKDAWN HQEFLEGRIL SEQKFKAHPN DVFLASYPKS GTTWLKAW♦I CIITREKFDD STSPLLTTMP HDCIPLLEKD
pFST4'   (1-110)      METTKTQFES -AEM--K---   ---S--G-I -----Q-F-G L-NNI--A-- AQ-S---R-D ----C----- ---------Y --V------E F------NI- -N---YI---
pHeST    (1-81)       ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦MSDD- LWFEGIAFPT MGFRS-TLRK VRDE-VIRDE --IILT---- --N--AEILC LMHSKGDAKW IQ-VPI♦♦♦♦ WERS-WV-SE
pHhST    (1-81)       ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦MSDD- LWFEGIAFPT MGFRS-TLRK VRDE-VIRDE --IILT---- --N--AEILC LMHSKGDAKW IQ-VPI♦♦♦♦ WERS-WV-SE
pMST     (1-90)       ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦EFPY KELFMMMSDY NWFEGIPFPA ISYQR-ILED IRNK-VVKEE -LLILT---- --N--IEIVC L-Q-KGDPKW IQTVPI♦♦♦♦ WNRS-WI-T-
pRST     (1-80)       ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦MPDY -WFEGIPFHA FGISK-TLQN VCNK-VVKEE -LI-LA---- --N--IEIVC L-Q-KGDPKW IQ-VTI♦♦♦♦ WDRS-WI-T-
pHaST-a  (1-85)       ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦♦♦ME LIQDTSRPPL EYV-GVPL♦♦ IKY-A-ALGP L♦-S-Q-R-D -LLIST---- ----VSQILD M-YQGGDLEK CHRAPI♦♦♦♦ FMRV-F--FK
pHpST    (1-85)       ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦♦♦ME LIQDTSRPPL EYV-GVPL♦♦ IKY-A-ALGP L♦-S-Q-R-D -LLIST---- ----VSQILD M-Y GDLEK CHRAPI♦♦♦♦ FMRV-F--FK
pHaST-b  (1-85)       ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦♦♦ME LIQDTSRPPL EYV-GVPL♦♦ IKY-A-ALGP L♦-S-Q-R-D -LLINT---- ----VSQILD M-\ GDLEK CNRAPI♦♦♦♦ YVRV-F--VN
pRmST    (1-81)       ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦♦♦ME F♦♦♦♦SRPPL VHV-GIPL♦♦ IKY-A-TIGP L♦-N-T-W-D -LLIST---- ----MSEILD M-YQGGKLEK CGRAPI♦♦♦♦ YARV-F--FK
pBeST    (1-85)       ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦♦♦MS SSKPSFSDY- GKLGGIPM♦♦ YKK-I-QFHN V♦EE-E-R-D -LVIVT---- -----SEIIC M-YNNGDVEK CKEDVI♦♦♦♦ FNRV-Y--CS
pReST    (1-85)       ♦♦♦♦♦♦♦♦♦♦ ♦♦♦♦♦♦♦♦ME TSMPEYYDV- GDFHGFLM♦♦ DKR-TKYWED V♦E--L-R-D -LLIVT---- -S--ISEIVD M-YKEGDVEK CKEDA-♦♦♦♦ FNR--D--CR

pFST3    (100-205)     LEKIQEN♦♦♦ ♦QRNSLYTPI STHFHYKSLP ESARTSNCKI VYIYRNMKDV IVSYYHFLRQ IVKLSVEEAP FEEAFDEFCQ GISSCGPYWE HIKGYWKASL EKPEIFLFLK
pFST4'   (111-216)     -K--V--♦♦♦ ♦-N--CF--M A--MP-HV-- K-ILAL---M ------I--- ---F---G-E -T--PL-D-- --------YH ---QF----D -L-------- -R--VI----
pHeST    (82-183)      I♦♦♦♦♦GYTA LSESESPRLF -S-LPIQLF- K-FFS-KA-V I-LM--PR-- L--G-F-♦WK NM-FIKKPKS W--YFEW--- -TVLY-SWFD --H-WM♦♦PM REEKN--L-S
pHhST    (82-183)      I♦♦♦♦♦GYTA LSETESPRLF -S-LPIQLF- K-FFS-KA-V I-LM--PR-- L--G-F-♦WK NM-FIKKPKS W--YFEW--- -TVVY-SWFD --H-WM♦♦PM REEKN--L-S
pMST     (91-192)      I♦♦♦♦♦GYSA LINKEGPRL- TS-LPIHLFS K-FFS-KA-A I-LV--PR-I L--G-F-♦WG NTN-VKNPGS LGTYFEW-LK -NVLF-SWF- -VR-WL♦♦-M REWDN--V-Y
pRST     (81-182)      V♦♦♦♦♦GYDI LIKKKGPRLM TS-LPMHLFS K-LFS-KA-V I-LI--PR-- L--G-Y-♦WG NST-VKKPDS LGTY-EW-LK -NVLY-SWF- --RAWL♦♦-M REWDH--L-Y
pHaST-a  (86-192)      APG-PSGMET LKDTPAPRLL K--LPLAL-- QTLLDQKV-V --VA--A--- A------♦YH MA-VHP-PGT WDSFLEK-MV -EV-Y-SWYQ -VQEW-♦♦E- SRTHPV-Y-F
pHpST    (86-192)      APG-PSGMET LKDTPAPRLL K--LPLAL-- QTLLDQKV-V --VA--A--- A------♦YH MA-VHP-PGT WDSFLEK-MV -EV-Y-SWYQ -VQEW-♦♦E- SRTHPV-Y-F
pHaST-b  (86-192)      DPGEPSGLET LKDTPPPRL- KS-LPLAL-- QTLLDQKV-V --VA--A--- A------♦HR ME-AHP-PGT WDSFLEK-MA -EV-Y-SWYQ -VQEW-♦♦E- SRTHPV-Y-F
pRmST    (82-188)      CPGVPSGLET LEETPAPRLL K--LPLSL-- Q-LLDQKV-V I--A--A--- V----N-♦YN MA--HPDPGT WDSFLEN-MD -EV-Y-SWYQ -VKEW-♦♦E- RHTHPV-Y-F
pBeST    (86-192)      T-HVMKGVKQ LNEMASPRIV KS-LPV-L-- V-FWEK---- I-LS--A--- V----FL♦IL M-TAIPDPDS -QDF-EK-MD -EVPY-SWF- -TKSW-♦♦EK S-MPQV---F
pReST    (86-192)      N-DLINGIKQ LKEKESPRIV K--LPA-L-- A-FWEK---- I-LC--A--- V----Y-♦FL -M-SYPNPKS -S-F-EK-ME -QVPY-SWYD -VKSW-♦♦EK S-NSRV--MF

                                                                                                    RKGXXGDWKN XFT
pFST3    (206-311)     YEDMKKDPVP SVKKLADFIG HPFTPKEEEA GVIEDIVKLC SFEKLSSLEV NKSGMHRPEE AHSIENRLYF RKGKDGDWKN YFTDEMTQKI DKLIDEKLGA TGLVLK*
pFST4'   (217-320)     ---V----TS N--R--E--- Y---FE--KE ----S----- ---N--N--- ----♦♦NSKG FLP------- --A------- ---------- --------S- ------*
pHeST    (184-285)     --EL-Q-TGR TIE-ICQ-L- KTLE-E-♦♦♦ ♦♦LNL-L-NS --QSMKENKM SNYSLL♦SVD YVVDKA♦QLL ---VS----- H--VAQA-DF ---FQ--MAD ♦♦♦♦-PRELF
pHhST    (184-285)     --EL-Q-TGR TIE-ICQ-L- KTLE-E-♦♦♦ ♦♦LNL-L-NS --QSMKENKM SNYSLL♦SVD YVVDKA♦QLL ---VS----- H--VAQA-DF ---FQ--MAD ♦♦♦♦-PRELF
pMST     (193-294)     ---I---TKG TI--ICD-L- KNLG-D-♦♦♦ ♦♦LDLVL-YS --QAMKENNM SNFSLI♦KED QVTNGL♦KLM ---TI----- H--VAQA-AF --VFQ--MAG ♦♦♦♦FPPGIF
pRST     (183-284)     -------TMG TI--IC--L- KKLE-D-♦♦♦ ♦♦LDLVL-YS --QVMKENDM SNYSLL♦MKK SIFTGI♦GLM ---TI----- H--VSQA-AF --VFQ--MAG ♦♦♦♦FPPPGMF
pHaST-a  (193-294)     -----EN-KR EIQ-ILE-V- -SLPEET♦♦♦ ♦♦VDFMVQHT --KEMKKNPM TNYTTV♦-Q- FMDHSISPFM ---MA----T T--VAQN-RF -ADYA--MAG CS-SFRSEL*
pHpST    (193-294)     -----EN-KR EIQ-ILE-V- RSLPEET♦♦♦ ♦♦VDFMVQHT --KEMKKNPM TNYTTV♦-Q- FMDHSISPFM ---MA----T T--VAQN-RF -ADYAK-MAG CS-TFRSEL*
pHaST-b  (193-294)     -----EN-KR EIQ-ILE-V- RSLPEET♦♦♦ ♦♦MDFMVQHT --KEMKKNPM TNYTTV♦-Q- LMDHSISPFM ---MA----T T--VAQN-RF -ADYA--MAG CS-SFRSEL*
pRmST    (189-290)     ---I-EN-KR EI--ILE-L- RSLPEET♦♦♦ ♦♦VDS-VHHT --KKMKENCM TNYTTI♦-T- IMDHNVSPFM ---TT----- T--VAQN-RF -AHYAKTMTD CDFKFRCEL*
pBeST    (193-294)     -----ENIRK E-M--LE-L- RKASDEL♦♦♦ ♦♦VDK---HT --QEMKNNPS TNYTTL♦-D- VMNQKVSPFM -- DV----- H--VALN--F -MHYEQQMKG ST-KFRTKI*
pReST    (193-294)     -----E-IRR E-V--IE-LE RDPSAEL♦♦♦ ♦♦VDR--QHT --QEMKNNPC TNYS-L♦-ET MIDLKVSPFM ---IV----- H-PEALR-RF EEHYQQQMKD CPVKFRAEL*
```

STs (Fig. 3) were speculated to be related to the PAPS binding sites. Conserved amino acid residues 291-303, containing a GXXG motif have been proposed to be a PAPS binding site (Hashimoto et al., 1992; Zhu et al., 1993; Weinshilboum and Otterness, 1994). So far, no experimental evidence has confirmed the PAPS binding sites of STs to be similar to those of ATP- and GTP-binding proteins. Moreover, other nucleotide-bearing enzymes contain different motifs for nucleotide-binding sites. For example, the nucleotide binding site of nucleoside diphosphate kinases is located at a turn of a distorted helix between ß2 and α2 (Dumas et al., 1992; Moréra et al., 1994) which is quite distinct from a classical mononucleotide binding site. Thus, more evidence would be required to deduce the PAPS binding site.

## D. Literature cited

Akeda S, Kung S, Dube SK (1993a) Nucleotide sequence of a soybean chalcone synthase gene with a possible role in ultraviolet-B sensitivity, Gmchs6. Plant Physiol 102: 699-701

Akeda S, Kung S, Dube SK (1993b) Nucleotide sequence and putative regulatory elements of gene 2 of the soybean (Glycine max) chalcone synthase multigene family. Plant Physiol 102: 317-319

Akeda S, Kung S, Dube SK (1993c) Nucleotide sequence and putative regulatory elements of a nodule-development-specific member of the soybean (Glycine max) chalcone synthase multigene family, Gmschs7. Plant Physiol 102: 317-319

An C, Ichinose Y, Yamada T, Tanaka Y, Shiraishi T, Oku H (1993) Organization of the gene encoding chalcone synthase in Pisum sativum. Plant Mol Biol 21: 789-803

Anson JG, Gilbert HJ, Oram JD, Minton NP (1987) Complete nucleotide sequence of the Rhodosporidium toruloides gene coding for phenylalanine ammonia-lyase. Gene 58: 189-199

Arioli T, Howles PA, Weinman JJ, Rolfe BG (1994) In Trifolium subterraneum, chalcone synthase is encoded by a multigene family. Gene 138: 79-86

Barron D, Varin L, Ibrahim RK (1986) Identification and biosynthesis of glucosylated and sulfated flavonols in Flaveria bidentis. Z Naturforsch 41c: 813-819

Barron D, Varin L, Ibrahim RK, Harborne JB, William CA (1988) Sulfated flavonoids- an update. Phytochemistry 27: 2375- 2395

Batschauer A, Ehmann B, Schafer E (1991) Cloning and characterization of a chalcone synthase gene from mustard and its light-dependent expression. Plant Mol Biol 16: 175-185

Bell EA (1981) The physiological role(s) of secondary (natural) products. In PK Stumpf, EE Conn, eds, The Biochemistry of Plants: a Comprehensive Treatise, Vol 7. Academic Press, New York, pp 1-20

Bernier F, Solache LI, Labrie F, Luu-The V (1994) Cloning and expression of cDNA encoding human placental estrogen sulfotransferase. Mol Cell Endocrin 99: R11-R15

Bloksberg LN, Kado CI (1991) A tomato phenylalanine ammonia-lyase gene includes RNA editing and a transcriptional regulator in the protein coding sequence. EMBL database accession number M83314 ID TOMPHEAMLY

Bowler C, Neuhaus G, Yamagata H, Chua N (1994) Cyclic GMP and calcium mediate phytochrome phototransduction. Cell 77: 73-81

Brandan E, Hirschberg CB (1988) Purification of rat liver N-heparan-sulfate sulfotransferase. J Biol Chem 263: 2417-2422

Chamberland H, Lafontaine J, Marsolais F, Richard M, Varin L (1994) Characterization of a tissue-specific, sulfotransferase-like protein in Mimosa pudica. Groupe Polyphenols L15

Chapple CCS, Vogt T, Ellis BE, Somerville CR (1992) An Arabidopsis mutant defective in the general phenylpropanoid pathway. Plant Cell 4: 1413-1424

Comer KA, Falany JL, Falany CN (1993) Cloning and expression of human liver dehydroepiandrosterone sulfotransferase. Biochem 289: 233-240

Cone KC, Cocciolone SM, Burr FA, Burr B (1993a) Maize anthocyanin regulatory gene pl is a duplicate of c1 that functions in the plant. Plant Cell 5: 1795-1805

Cone KC, Cocciolone SM, Moehlenkamp CA, Weber T, Drummond BJ, Tagliani LA, Bowen BA, Perrot GH (1993b) Role of the regulatory gene pl in the photocontrol of maize anthocyanin pigmentation. Plant Cell 5: 1807-1816

Cramer CL, Edwards K, Dron M, Liang X, Dildine SL, Bolwell GP, Dixon RA, Lamb CJ, Schuch W (1989) Phenylalanine ammonia-lyase gene organization and structure. Plant Mol Biol 12: 367-383

Dalkin K, Edwards R, Edington B, Dixon RA (1990) Stress responses in Alfalfa (Medicago sativa L.) I. Induction of phenylpropanoid biosynthesis and hydrolytic enzymes in elicitor-treated cell suspension cultures. Plant Physiol 92: 440-446

Dangl JL, Hahlbrock K, Scheel J (1989) Regulation and structure of chalcone synthase genes. In J Scheel, IK Vasil, eds, Cell Culture and Somatic Cell Genetics of Plants, Vol 6. Academic Press,

Inc., San Diego, pp 155-173

Demyan WF, Song CS, Kim DS, Her S, Gallwitz W, Rao TR, Slomczynska M, Chatterjee B, Roy AK (1992) Estrogen sulfotransferase of the rat liver: complementary DNA cloning and age-specific regulation of messenger RNA. Mol Endocrin 6: 589-597

Dixon RA (1986) The phytoalexin response: elicitation, signalling and control of host gene expression. Biol Rev 61: 239-291

Dixon RA, Dey PM, Lamb CJ (1983) Phytoalexins: enzymology and molecular biology. Adv Enzymol Relat Areas Mol Biol 55: 1-135

Djordjevic MA, Redmond JW, Batley M, Rolfe BG (1987) Clovers secret compounds which either stimulate or repress nod gene function in Rhizobium trifolii. EMBO J 6: 1173-1179

Driscoll WJ, Martin BM, Chen H, Stroll CA (1993) Isolation of two distinct 3-hydroxysteroid sulfotransferases from the guinea pig adrenal gland; evidence for 3α-hydroxy versus 3ß-hydroxy steroid stereospecificity. J Biol Chem 268: 23496-23503

Dumas C, Lascu I, Moréra S, Glaser P, Fourme R, Wallet V, Lacombe M, Véron M, Janin J (1992) X-ray structure of nucleoside diphosphate kinase. EMBO J 11: 3203-3208

Edwards K, Cramer CL, Bolwell GP, Dixon RA, Schuch W, Lamb CJ (1985) Rapid transient induction of phenylalanine ammonia-lyase mRNA in elicitor-treated bean cells. Proc Natl Acad Sci USA 82: 6731-6735

Edwards R, Dixon R (1991) Isoflavone O-methyltransferase activities in elicitor-treated cell suspension cultures of Medicago sativa. Phytochemistry 30: 2597-2606

Eriksson I, Sandbäck D, Ek B, Lindahl U, Kjelén L (1994) cDNA cloning and sequencing of mouse mastocytoma glucosaminyl N-deacetylase/N-sulfotransferase, an enzyme involved in the biosynthesis of heparin. J Biol Chem 269: 10438-10443

Estabrook EM, Sengupta-Gopalan C (1991) Differential expression of phenylalanine ammonia-lyase and chalcone synthase during soybean nodule development. Plant Cell 3: 299-308

Faulkner IJ, Rubery PH (1992) Flavonoids and flavonoid sulfates as probes of auxin-transport regulation in Curcubita pepo hypocotyl segments and vesicles. Planta 186: 618-625

Feinbaum RL, Ausubel FM (1988) Transcriptional regulation of the Arabidopsis thaliana chalcone synthase gene. Mol Cell Biol 8: 1985-1992

Filpula D, Strausberg RL, Vaslet CA, Sykes A, Levy A (1988) Nucleotide sequence of a gene for phenylalanine ammonia-lyase from Rhodotorula rubra. Nucleic Acids Res 16: 11381

Fisher RF, Long SR (1992) Rhizobium-plant signal exchange. Nature 357: 655-660

Firmin JL, Wilson KE, Rossen L, Johnson AWB (1986) Flavonoid activation of nodulation genes in Rhizobium reversed by other compounds present in plants. Nature 324: 90-92

Foster R, Izawa T, Chua NH (1994) Plant bZIP proteins gather at ACGT elements. FASEB J 8: 192-200

Frank RL, Vodkin LO (1991) Sequence and structure of a phenylalanine ammonia-lyase gene from Glycine max. DNA Seq 1: 335-346

Glendening TM, Poulton JE (1988) Glucosinolate biosynthesis: sulfation of desulfobenzylglucosinolate by cell-free extract of cress (Lepidium sativum L.) seedlings. Plant Physiol 86: 319-321

Gowri G, Paiva NL, Dixon RA (1991) Stress responses in alfalfa (Medicago sativa L.) 12. Sequence analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor-treated cell cultures and developing plants. Plant Mol Biol 17: 415-429

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol 40: 347-369

Hanks S, Quinn AM, Hunter T (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241: 42-52

Hannoufa A, Varin L, Ibrahim RK (1991) Spatial distribution of flavonoid conjugates in relation to gulcosyltransferase and sulfotransferase activities in Flaveria bidentis. Plant Physiol 97: 259-263

Hannoufa A, Brown RH, Ibrahim RK (1994) Variations in flavonoid sulfate patterns in relation to photosynthesis types of five Flaveria species. Phytochemistry 36: 353-356

Hanson KR, Havir EA (1981) Phenylalanine ammonia-lyase. In PK Stumpf, EE Conn, eds, The Biochemistry of Plants: a Comprehensive Treatise, Vol 7. Academic Press, New York, pp 577-626

Harborne JB (1975) Flavonoid sulfate: a new class of sulfur compounds in higher plants. Phytochemistry 14: 1147-1155

Harborne JB (1988) The flavonoids: advance in research since 1980. Champman and Hall, London.

Harker CL, Ellis THN, Coen ES (1990) Identification and genetic regulation of the chalcone synthase multigene family in pea. Plant Cell 2: 185-194

Harter K, Kircher S, Frohnmeyer H, Krenz M, Nagy F, Schafer E (1994) Light-regulated modification and nuclear translocation of cytosolic G-box binding factors in parsley. Plant Cell 6: 545-559

Hashimoto Y, Orellana A, Gil G, Hirschberg CB (1992) Molecular cloning and expression of rat liver N-heparan sulfate sulfotransferase. J Bio Chem 267: 15744-15750

Heller W, Forkmann G (1988) Biosynthesis. In JB Harborne, ed, The Flavonoids. Chapmann and Hall Ltd., pp 399-425

Hellen RP (1994) Pisum sativum gene for chalcone synthase. EMBL accession number X80007 ID PSCHSAB

Herrmann A, Schulz W, Hahlbrock K (1988) Two alleles of the single-copy chalcone synthase gene in parsley differ by a transposon-like element. Mol Gen Genet 212: 93-98

Howles PA, Arioli T, Weinman JJ (1994) Characterization of a phenylalanine ammonia-lyase multigene family in Trifolium subterraneum. Gene 138: 877-892

Huttner WB (1988) Tyrosine sulfation and the secretory pathway. Ann Rev Physiol 50: 363-376

Ibrahim RK, De Luca V, Khouri H, Latchinian L, Brisson L, Charest (1987) Enzymology and compartmentation of polymethylated flavonol glucosides in Chrysosplenium americanum. Phytochemistry 26: 1237- 1245

Ibrahim RK, Varin L (1993) Flavonoid enzymology. In PJ Lea, ed, Methods in Plant Biochemistry, Vol 9, Academic Press, New York, pp 99-129

Ichinose Y, Kawamata S, Yamada T, Kajiwara T, Shiraishi T, Oku H (1992) Molecular cloning of chalcone synthase cDNAs from Pisum sativum. Plant Mol Biol 18: 1009-1012

Ishihara M, Guo Y, Wei Z, Yang Z, Swiedler SJ, Orellana A, Hirschberg CB (1993) Regulation of biosynthesis of the basic fibroblast growth factor binding domain of heparan sulfate by heparan sulfate-N-deacetylase/N-sulfotransferase expression. J Biol Chem 268: 20091-20095

Jacobs M, Rubery PH (1988) Naturally occurring auxin transport regulators. Science 241: 346-349

Jain JC, GrootWassink JWD, Kolenovsky AD, Underhill EW (1990) Purification & ' properties of 3'-phosphoadenosine-5'-phosphosulfate: desulfoglucosinolate sulfotransferase from Brassica juncea cell culture ytochemistry 29: 1425-1428

Joos H, Hahlbrack K (1992) Phenylalanine ammonia-lyase in potato (Solanum tuberosum L.) Eur J Biochem 204: 621-629

Junghans H, Dalkin K, Dixon RA (1993) Stress responses in alfalfa (Medicago sativa) XV Characterization and expression patterns of members of a subset of the chalcone synthase multigene family. Plant Mol Biol 22: 239-253

Kawamata S, Yamada T, Tanaka Y, Sriprasertsak P, Kato H, Ichinose Y, Kato H, Shiraishi T, Oku H (1992) Molecular cloning of phenylalanine ammonia-lyase cDNA from Pisum sativum. Plant Mol Biol 20: 167-170

Khan AS, Taylor BR, Chung K, Etheredge J, Gonzales, Ringer DP (1993) Genomic structure of rat liver aryl sulfotransferase IV-encoding gene. Gene 137: 321-326

Khouri HE, Tahara S, Ibrahim RK (1988) Partial purification, characterization, and kinetic analysis of isoflavone 5-O-methyltransferase from yellow lupin roots. Arch Biochem Biophys 262: 592-598

Kobayashi T, Honke K, Kamino K, Sakakibara N, Gasa S, Miyao N, Tsukamoto T, Ishizuka I, Miyazaki T, Makita A (1993) Sulfolipids and glycolipids sulfotransferase activities in human renal cell carcinoma cells. J Cancer 67: 76-80

Koes RE, Spelt CE, van den Elzen PJM, Mol JNM (1989) Cloning and molecular characterization of the chalcone synthase multigene family of Petunia hybrida. Gene 81: 245-257

Koes RE, Quattrocchio F, Mol JNM (1994) The flavonoid biosynthesis pathway in plants: function and evolution. Bioassays 16: 123-132

Kong AT, Yang L, Ma M, Tao D, Bjornsson TD (1992) Molecular cloning of the alcohol/hydroxysteroid form ($hST_\beta$) of sulfotransferase from human liver. Biochem Biophys Res Commun 187: 448-454

Kong AT, Ma M, Tao D, Yang L (1993) Molecular cloning of cDNA encoding the phenol/aryl form of sulfotransferase ($mST_{p1}$) from mouse liver. Biochim Biophys Acta **1171**: 315-318

Kosslak RM, Bookland R, Barkei J, Paaren HE, Appelbaum ER (1987) Induction of Bradyrhizobium japonicum common nod genes by isoflavones isolated from Glycine max. Proc Natl Acad Sci USA **84**: 7428-7432

Kubassek WL, Shirley BW, McKillop A, Goodman HM, Briggs W, Ausubel FM (1992) Regulation of flavonoid biosynthetic genes in germinating Arabidopsis seedling. Plant Cell **4**: 1229-1238

Lacombe C, Roby D (1993) Arabidopsis transcribed sequence (RaR047) 5'-end similar to flavonol 4-sulfotransferase of Flaveria chloraefolia. EMBL accession number Z23001 ID ATTS0905

Lamb CJ, Lawton MA, Dron M, Dixon RA (1989) Signals and transduction mechanisms for activation of plant defense against microbial attack. Cell **56**: 215-224

Lawton MA, Dixon RA, Hahlbrock K, Lamb CJ (1983) Elicitor induction of mRNA activity: Rapid effects of elicitor on phenylalanine ammonia-lyase and chalcone synthase mRNA activities in bean cells. Eur J Biochem **130**: 131-139

Latchinian L, Khouri HE, Ibrahim RK (1987) Fast protein affinity chromatography of two flavonoid glucosyltransferases. J Chrmatography **388**: 235-242

Leitinger B, Brown J, Spiess M (1994) Tagging secretory and membrane proteins with a tyrosine sulfation site. J Biol Chem **269**: 8115-8121

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Promé JC, Dénarié J (1990) Symbiotic host-specificity of Rhizobium meliloti is determined by a sulphated and acetylated glucosamine oligosaccharide signal. Nature **344**: 781-784

Leyva A, Liang X, Pintor-Toro JA, Dixon RA, Lamb CJ (1992) Cis-element combinations determine phenylalanine ammonia-lyase gene tissue-specific expression patterns. Plant Cell **4**: 263-271

Li J, Ou-Lee T, Raba R, Amundson RG, Last RL (1993) Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell **5**: 171-179

Liang X, Dron M, Cramer CL, Dixon RA, Lamb CJ (1989a) Differential regulation of phenylalanine ammonia-lyase genes during plant development and by environmental cues. J Biol Chem **264**: 14486-14492

Liang X, Dron M, Schmid J, Dixon RA, Lamb CJ (1989b) Developmental and environmental regulation of a phenylalanine ammonia-lyase-β-glucuronidase gene fusion in transgenic tobacco plants. Proc Natl Acad Sci USA **86**: 9284-9288

Loake GJ, Choudhary AD, Harrison MJ, Mavandad M, Lamb CJ, Dixon RA (1991) Phenylpropanoid pathway intermediates regulate transient expression of a chalcone synthase gene promoter. Plant Cell **3**: 829-840

Loake GJ, Faktor O, Lamb CJ, Dixon RA (1992) Combination of H-box [CCTACC(N)$_7$CT] and G-box (CACGTG) cis elements is necessary for feed-forward stimulation of a chalcone synthase promoter by the phenylpropanoid-pathway intermediate p-coumaric acid. Proc Natl Acad Sci USA **89**: 9230-9234

Lois R, Dietrich A, Hahlbrock K, Schulz W (1989) A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive cis-acting elements. EMBO J 8: 1641-1648

Long S (1989) Rhizobium-legume nodulation: life together in the underground. Cell 56: 203-214

Lynn DG, Chang M (1990) Phenolic signals in cohabitation: implications for plant development. Annu Rev Physiol Plant Mol Biol 41: 497-526

Mann J (1987) Secondary Metabolism, Second Edition, Clarendon Press, Oxford

Mavandad M, Edwards R, Liang X, Lamb CJ, Dixon RA (1990) Effects of trans-cinnamic acid on expression of the bean phenylalanine ammonia-lyase gene family. Plant Physiol 94: 671-680

McCombie WR, Kelley JM, Aubin L, Goscoechea M, FitzGerald MG, Wu A, Adams MD, Dubnick M, Kerlavage AR (1992) Caenorhabditis elegans cDNA clone CEESY22 similar to heparan sulfate sulfotransferase. EMBL accession number T02006 ID WEST02727

McKhann HI, Hirsch AM (1994) Isolation of chalcone synthase and chalcone isomerase cDNAs from alfalfa (Medicago sativa L.): highest transcript levels occur in young roots and root tips. Plant Mol Biol 24: 767-777

Meyerowitz EM (1989) Arabidopsis, a useful weed. Cell 56: 263-269

Miller RR, Waechter CJ (1988) Partial purification and characterization of detergent-solubilized N-sulfotransferase activity associated with calf brain microsome. J Neurochem 51: 87-94

Mo Y, Nagel C, Taylor LP (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. Proc Natl Acad Sci USA 89: 7213-7217

Moniz de Sa M, Subramaniam R, William FE, Douglas CJ (1992) Rapid activation of phenylpropanoid metabolism in elicitor-treated hybrid poplar (Populus trichocarpa x P. deltoides) suspension cultured cells. Plant Physiol 98: 728-737

Moréra S, Lascu I, Dumas C, LeBras G, Briozzo P, Véron M, Janin J (1994) Adenosine 5'-diphosphate binding and the active site of nucleoside diphosphate kinase. Biochemistry 33: 459-467

Nagai N, Kitauchi H, Shimosaka M, Okazaki M (1994) Cloning and sequencing of a full length cDNA coding for phenylalanine ammonia-lyase from tobacco cell culture. Plant Physiol 104: 1091-1092

Nagata K, Ozawa S, Miyata M, Shimada M, Gong D, Yamazoe Y, Kato R (1993) Isolation and expression of a cDNA encoding a male-specific rat sulfotransferase that catalyses activation of N-hydroxy-2-acetylaminofluorene. J Biol Chem 268: 24720-24725

Nash AR, Glenn WK, Moore SS, Kerr J, Thompson AR, Thompson EOP (1988) Oestrogen sulfotransferase: molecular cloning and sequencing of cDNA for the bovine placental enzyme. Aust J Biol Sci 41: 507-516

Oeda T, Lee YC, Driscoll WJ, Chen H, S rott CA (1992) Molecular cloning and expression of a full length complementary DNA encoding the guinea pig adrenocortical estrogen sulfotransferase. Mol

Endocrin 6: 1216-1226

Ogura K, Kajita J, Narihata H, Watabe T, Ozawa S, Nagata K, Yamazoe Y, Kato R (1990a) Cloning and sequence analysis of a rat liver cDNA encoding hydroxysteroid sulfotransferase. Biochem Biophys Res Commun 165: 168-174

Ogura K, Kajita J, Narihata H, Watabe T, Ozawa S, Nagata K, Yamazoe Y, Kato R (1990b) cDNA cloning of the hydroxysteroid sulfotransferase STa sharing a strong homology in amino acid sequence with the senescence marker protein SMP-2 in rat liver. Biochem Biophys Res Commun 166: 1494-1500

Ohl S, Hedrick SA, Chory J, Lamb CJ (1990) Functional properties of a phenylalanine ammonia-lyase promoter from Arabidopsis. Plant Cell 2: 837-848

Orellana A, Hirschberg CB, Wei Z, Swiedler SJ, Ishihara M (1994) Molecular cloning and expression of a glycosaminoglycan N-acetylglucosaminyl N-deacetylase/N-sulfotransferase from a heparin-producing cell line. J Biol Chem 269: 2270-2276

Orr JD, Edwards R, Dixon RA (1993) Stress responses in alfalfa (Medicago sativa L.) XIV. Changes in the levels of phenylpropanoid pathway intermediates in relation to regulation of L-phenylalanine ammonia-lyase in elicitor-treated cell suspension cultures. Plant Physiol 101: 847-856

Osakabe Y, Ohtsubo Y, Kawai S, Katayama Y, Morohoshi N (1994) Structures and tissue-specific expression of phenylalanine ammonia-lyase genes from a hybrid aspen, Populus kitakamiensis. EMBL accession number D30657 ID POPALB, 30656 ID POPPALA

Otterness DM, Wieben ED, Wood TC, Watson RWG, Madden BJ, McCormick DJ, Weinshilboum RM (1992) Human liver dehydroepiandrosterone sulfotransferase: molecular cloning and expression of cDNA. Mol Pharmacol 41: 865-872

Ozawa S, Nagata K, Gong D, Yamazoe Y, Kato R (1990) Nucleotide sequence of a full-length cDNA (PST-1) for a yl sulfotransferase from rat liver. Nucleic Acids Res 18: 4001

Paul SM, Purdy RH (1992) Neuroactive steroids. FASEB J 6: 2311-2322

Pollak PE, Vogt T, Mo Y, Taylor L (1993) Chalcone synthase and flavonol accumulation in stigma and anthers of Petunia hybrida. Plant Physiol 102: 925-932

Quattrocchio F, Wing JF, Leppen HTC, Mol JNM, Koes RE (1993) Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct set of target genes. Plant Cell 5: 1497-1512

Rasmussen OF, Orum H (1991) Analysis of the gene for phenylalanine ammonia-lyase from Rhodosporidium toruloides. DNA Seq 1: 207-211

Redmond JW, Batley M, Djordjevic MA, Innes RW, Kuempel PL, Rolfe BG (1986) Flavones induce expression of nodulation genes in Rhizobium Nature 323: 632-634

Reimold U, Kroeger M, Kreuzaler F, Hahlbrock K (1983) Coding and 3' non-coding nucleotide sequence of chalcone synthase mRNA and assignment of amino acid sequence of the enzyme. EMBO J 2: 1801-1805

Rohde WE, Dorr R, Salamini F, Becker D (1991) Structure of a chalcone synthase gene from Hordeum vulgare. Plant Mol Biol 16: 1103-1106

Roy AB (1981) Sulfotransferases. In GJ Mulder, ed, Sulfation of Drugs and Related Compounds, CRC Press, Boca Raton, pp 83-130

Ryder TB, Hedrick SA, Bell JN, Liang X, Clouse SD, Lamb CJ (1987) Organization and differential activation of a gene family encoding the plant defense enzyme chalcone synthase in Phaseolus vulgaris. Mol Gen Genet 210: 219-233

Sakac D, Zachos M, Lingwood CA (1992) Purification of testicular galactolipid: 3'phosphoadenosine 5'-phosphosulfate sulfotransferase. J Biol Chem 267: 1655-1659

Schildknecht H, Orson ER, Foster VE, Kosslak RM, Verma DPS (1990) Role of turgorins in leaf movement. In RL Satter, HL Gorton, TC Vogelmann, eds, The Pulvinus: Motor Organ for Leaf Movement. The American Society of Plant Physiologists, Rockville, MD, pp 205-213

Schmid J, Doerner PW, Clouse SD, Dixon RA, Lamb CJ (1990) Developmental and environmental regulation of a bean chalcone synthase promoter in transgenic tobacco. Plant Cell 2: 619-631

Schulze-Lefert P, Becker-Andree M, Schulz W, Hahlbrock K, Dangl JL (1989) Functional architecture of the light-responsive chalcone synthase promoter from parsley. Plant Cell 1: 707-714

Shieh M, Spear P (1994) Herpesvirus-induced cell fusion that is dependent on cell surface heparan sulfate or soluble heparin. J Virol 68: 1224-1228

Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C (1994) Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (Vitis vinifera L.) Plant Mol Biol 24: 743-755

Stafford HA (1990) Flavonoid Metabolism, CRC Press, Inc., Boca Raton, Florida

Subramaniam R, Reinold S, Molitor E, Douglas CJ (1993) Structure, inheritance, and expression of hybrid poplar (Populus trichocarpa x P. deltoides) phenylalanine ammonia-lyase genes. Plant Physiol 102: 71-83

Tanaka Y, Matsuoka M, Yamanoto N, Ohashi Y, Murakami YK, Ozeki Y (1989) Structure and characterization of cDNA clone for phenylalanine ammonia-lyase from cut-injured roots of sweet potato. Plant Physiol 90: 1403-1407

TT Vo L, Broughton WJ, Krause A (1993) Symbiotically induced accumulation of chalcone synthase transcripts in Vigna unguiculata. EMBL accession number X74821 ID VUCHSCH

van der Goorbergh JA, Meerman JHN, de Wit H, Mulder GJ (1985) Reaction of 2-acetylaminofluorene-N-sulfate with RNA and glutathione: evidence for the generation of two reactive intermediates with different reactivities towards RNA and glutathione. Carcinogenesis 6: 1635-1640

van der Krol AR, Mur LA, de Lange P, Mol JNM, Stuitje AR (1990) Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effect. Plant

Mol Biol 14: 457-466

van der Meer IM, Stuitje AR, Mol JNM (1993) Regulation of general phenylpropanoid and flavonoid gene expression. In DPS Verma, ed, Control of Plant Gene Expression, CRC Press Inc, Boca Raton, pp 125-155

Varin L (1992) Flavonoid sulfation: phytochemistry, enzymology, and molecular biology. In HA Stafford, Ibrahim RK, eds, Phenolic Metabolism in Plants, Plenum Press, New York, pp 233-254

van der Meer IM, Stam M, van Tunen AJ, Mol JNM, Stuitje AR (1992) Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. Plant Cell 4: 253-262

Varin L, Barron D, Ibrahim RK (1986) Identification and biosynthesis of glucosylated and sulfated flavonols of Flaveria bidentis. Z Naturforsh 41c: 813-819

Varin L, Barron D, Ibrahim RK (1987a) Enzymatic synthesis of sulphated flavonols in Flaveria. Phytochemistry 26: 135-138

Varin L, Ibrahim RK (1989) Partial purification and characterization of three flavonol specific sulfotransferases from Flaveria chloraefolia. Plant Physiol 90: 977-981

Varin L, Ibrahim RK (1991) Partial purification and some properties of flavonol 7-sulfotransferase from Flaveria bidentis. Plant Physiol 95: 1254-1258

Varin L, Ibrahim RK (1992) Novel Flavonol 3-sulfotransferase: purification, kinetic properties and partial amino acid sequence. J Biol Chem 267: 1858-1863

Varin L, DeLuca V, Ibrahim RK, Brisson N (1992) Molecular characterization of two plant flavonol sulfotransferases (Flaveria chloraefolia). Proc Natl Acad Sci USA 89: 1286-1290

Vogt T, Pollak P, Tarlyn N, Taylor LP (1994) Pollination- or wound-induced kaempferol accumulation in petunia stigmas enhance seed production. Plant Cell 6: 11-23

Wanner LA, Li G, Ware D, Somssich IE, Davis KR (1994) The phenylalanine ammonia-lyase family in Arabidopsis thaliana. EMBL accession number L33677 ID ATHPAL, L33678 ID ATHPAL2, L33679 ID ATHPAL3

Weinshilboum R, Otterness D (1994) Sulfotransferase enzymes. In FC Kauffman, ed, Handbook of Experimental Pharmacology: Conjugation and Deconjugation Reaction in Drug Metabolism and Toxicity, Vol 112, Springer-Verlag, London, pp 45-78

Wilborn TW, Comer KA, Dooley TP, Reardon IM, Heinrinkson RL, Falany CN (1993) Sequence analysis and expression of the cDNA for the phenol-sulfating form of human liver phenol sulfotransferase. Mol Pharmacol 43: 70-77

Wood TC, Askoy IA (1994) Human liver thermolabile phenol sulfotransferase: cDNA cloning, expression and characterization. Biochem Biophys Res Commun 198: 1119-1127

Yamada T, Tanaka Y, Sriprasertsak P, Kato H, Hashimoto T, Kawamata S, Ichinose Y, Kato H, Shiraishi T, Oku H (1992) Phenylalanine ammonia-lyase genes from Pisum sativum: structure, organ-specific

expression and regulation by fungal elicitor and suppressor. Plant Cell Physiol 33: 715-725

Yerokun T, Etheredge JL, Norton TR, Carter HA, Chung KH, Birckbichler PJ, Ringer DP (1992) Characterization of a complementary DNA for rat liver aryl sulfotransferase IV and use in evaluating the hepatic gene transcript levels of rat at various stages of 2-acetylaminofluorene-induced hepatocarcinogenesis. Cancer Res 52: 4779-4786

Ylstra B, Touraev A, Moreno RMB, Stöger E, van Tunen AJ, Vicente O, Mol JNM, Heberle-Bors E (1992) Flavonols stimulate development, germination and tube growth of tobacco pollen. Plant Physiol 100: 902-907

Zhu X, Veronese ME, Sansom LN, McManus ME (1993a) Molecular characterization of a human aryl sulfotransferase cDNA. Biochem Biophys Res Commu 192: 671-676

Zhu X, Veronese ME, Bernard CCA, Sansom LN, McManus ME (1993b) Identification of two human brain aryl sulfotransferase cDNAs. Biochem Biophys Res Commu 195: 120-127

# CHAPTER II

## Effects of Nutrients, Phytohormones, Elicitors, and Flavonols on Growth and Flavonol Sulfotransferase Activity of *Flaveria bidentis* Cell Suspension Cultures

### A. Abstract

*Flaveria bidentis* cell suspension cultures consisting of small, uniform cell clusters were used to study the effects of nutrients, phytohormones, elicitors and flavonols on culture growth. In addition, flavonol 3-sulfotransferase (F3-ST) was characterized, and its activity was observed in response to different treatments. None of the nutrients or elicitors tested exhibited significant effects on F3-ST activity. The treatments with a synthetic auxin, 2,4-D showed an increase of F3-ST activity, whereas kinetin did not alter the level of F3-ST activity. Quercetin 3-sulfate showed an inhibitory effect on F3-ST activity. The possible physiological roles of sulfated flavonols are discussed.

### B. Introduction

Flavonoid compounds are of ubiquitous occurrence in plants and are known for their roles as flower pigments, and as stress metabolites formed in response to UV irradiation (Li *et al.*, 1993; Chapple *et al.*, 1992) and microbial attack (Lamb *et al.*, 1989), as regulators of auxin polar transport (Jacobs

and Rubery, 1988; Faulkner and Rubery, 1992), stimulators of pollen germination (Vogt et al., 1994; Pollak et al., 1993), and inducers of nodulation genes in Rhizobium-legume interaction (Long, 1989). In addition, they may play a role in the cohabitation of plants with the parasites, such as Striga species (Lynn and Chang, 1990). Flavonoid conjugates (methylated, glycosylated, prenylated and sulfated derivatives) have recently drawn much interest in the studies of signalling and regulation of plant-microbe interaction (Firmin et al., 1986; Djordjevie et al., 1987; Kosslak et al., 1987; Redmond et al., 1986), male sterility (van der Meer et al., 1992), and auxin polar transport (Jacobs and Rubery, 1988). Although its relation to phytoalexin synthesis is not yet clear, isoflavone *O*-methyltransferase of alfalfa cell cultures was reported to be induced ca 200-fold upon treatment with Colletotrichum lindemuthianum cell wall extract (Edwards and Dixon, 1991). Glucosylation and deglucosylation of the flavonol, kaempferol, have been proposed to control the level of pollen germination inducers (Pollak et al., 1993). Prenylation of isoflavones have been reported to confer resistance to fungal attack (Dixon et al., 1983). Flavonol sulfation has been proposed to be involved in the growth and development of Flaveria species (Hannoufa et al., 1991).

A number of position-specific flavonol sulfotransferases (F-STs) have recently been characterized from Flaveria spp., Asteraceae (Varin and Ibrahim, 1989; 1991). These enzymes

exhibit strict specificity for positions 3 of flavonol aglycones (F3-ST), 3' or 4' of flavonol 3-sulfate (F3'/F4'-ST) and 7 of flavonol 3,3'- or 3,4'-disulfates (F7-ST), thus establishing an enzymatic sequence for the formation of flavonol polysulfates (Varin, 1992). F. bidentis accumulates flavonol mono- to tetrasulfate esters (Barron et al., 1986) and exhibits several F-ST activities (Varin et al., 1987a). Although other position-specific F-STs of F. bidentis have not yet been thoroughly characterized, its F3-ST activity has recently been shown to be developmentally regulated, being highest in the shoot tips and the first pair of expanded leaves and lowest in mature leaves (Hannoufa et al., 1991). Therefore, a cell suspension culture of F. bidentis was established from terminal buds and used to study the effect of different factors on culture growth and F-ST activity. It has previously been reported that F. bidentis cell suspension cultures exhibited very low constitutive levels of F-ST activity and do not accumulate any flavonol sulfate esters (Bleichert et al., 1989).

In this study, we report the characterization of F3-ST of F. bidentis tissue cultures. The nutrient requirements of these cultures were determined in both MS and B5 culture media. The effects of nutrients, phytohormones, elicitors, and flavonols on growth and F3-ST activity were monitored following subculture in the conditioned media. Whereas none of the nutrients or elicitors tested had any significant

effect on F3-ST activity, the synthetic auxin, 2,4-D resulted in the induction of F3-ST activity. On the other hand, quercetin 3-sulfate, the F3-ST enzyme reaction product, exhibited an inhibitory effect on F3-ST activity. The possible roles of flavonol sulfates and their biosynthetic enzymes are discussed.

## C. Materials and Methods

### C.1 Plant material

Seeds of Flaveria bidentis var. angustifolia O.K. (Asteraceae) were germinated in vermiculite on top of potting soil, and plants were further propagated by cuttings. A callus culture of F. bidentis was initiated from terminal bud explants and maintained on a MS semi-solid medium containing 3% w/v sucrose, 4.5 $\mu$M 2,4-D and 0.45 $\mu$M kinetin (Bleichert et al., 1989). Calli were broken into small pieces and transferred to a liquid medium of the same composition. Growth of the cell suspension culture was maintained in the light, at room temperature, in 1-L nipple spherical flasks rotating centripetally at 4 rpm. Nipple flasks are near spherical with ten symmetrically spaced cylindrical protrusions, each approximately 2 cm long and wide. This type of flask increases the aeration of rotating cultures and, therefore, promotes culture growth (Steward et al., 1952). The cell cultures used in this study consisted of small,

uniform cell clusters as shown in Fig. 1.

**C.2 Chemicals**

PAP-agarose was obtained from Sigma (St. Louis, MO) and 3'-phosphoadenosine 5'-phospho-[$^{35}$S]sulfate (PAPS, 1.57 Ci/mmol) was from New England Nuclear (Boston, MA). Quercetin was purchased from Sigma and quercetin 3-sulfate was from Sarsynthèse (Mérignac, France). TBADP was obtained from Aldrich Chemical Co. (Milwaukee, WI), and the immunodetection kit from Bio-Rad (Mississauga, ON).

**C.3 Characterization of flavonol sulfotransferase activity of F. bidentis.**

Protein extracts from F. bidentis tissue cultures were subjected to partial purification and characterization of F3-ST activity using the method of Varin and Ibrahim (1992). Briefly, cell cultures were harvested and extracted with a buffer containing 100 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DIECA. The protein extract was fractionated with solid ammonium sulfate, and the protein which precipitated between 35 and 75% ammonium sulphate salt saturation was collected by centrifugation. The protein pellet was dissolved in 25 mM bis-Tris-HCl, pH 6.5 and desalted by passage through a PD-10 column (Pharmacia, ONT). The desalted protein was chromatographed on a PAP-agarose affinity column, previously equilibrated with 25 mM bis-Tris-HCl, pH 6.5. The bound

proteins were eluted in the same buffer using a salt gradient of 0.0 to 1.0 M NaCl. Fractions of one mL were collected and assayed for F-ST activity. The active fractions were pooled and concentrated using an Amicon cell concentrator (Amicon). The concentrated proteins were diluted in a buffer containing 25 mM bis-Tris-iminodiacetate (pH 7.5), and subjected to chromatofocusing on a Mono P column. The protein fractions were eluted with Polybuffer 74 (BioRad) which generated a gradient between pH 7.0 and 4.0. Fractions of one mL were collected in 0.3 mL buffer containing 200 mM Tris-HCl (pH 7.5) in order to stabilize F-ST activity. The F-ST active fractions were then subjected to western blot analysis. A polyclonal antiserum raised against F. chloraefolia F3-ST (Varin and Ibrahim, 1992) was used for the immunodetection of F-ST.

### C.4 Growth measurement of F. bidentis cell cultures

Cell culture growth was monitored by the changes in fresh weight, protein content, cell number and cell viability. An aliquot of cell suspension was collected by filtration through Whatman #1 filter paper using vacuum, and its fresh weight was calculated in gram per culture volume. Following extraction with buffer, the protein concentrations were determined according to the method of Bradford (1976) using the Bio-Rad reagent and BSA as the protein standard, and were expressed in mg protein per gram of fresh weight.

Cell viability was assessed by the use of Evans blue staining which was added to a final concentration of 0.023% w/v. After 5 min incubation, intact viable cells were counted under a light microscope. Dead cells appeared blue because of their inability to exclude the dye. Cell viability was presented as the number of viable cells as a function of the total cell count.

**C.5 Measurement of major nutrients in the cell culture medium**

MS (Murashige and Skoog, 1962) and B5 (Gamborg and Wetter, 1975) media were used for the measurement of nutrient requirement of F. bidentis plant cell cultures. Fresh media (250 mL in 1-L nipple flasks) were inoculated with 10 mL (ca 5 g fresh weight) of ten-day-old cell cultures. The media contained sucrose as the carbon source, inorganic nutrients, trace elements, and plant growth regulators. The depletion of most of these nutrients was monitored during the growth period.

**Sucrose.** The amount of sucrose in the culture medium was determined by the anthrone reaction after destruction of reducing sugars using a strong alkali (Handel, 1968). The sensitivity limit of such measurement was reported to be approximately 10 μg sucrose. Briefly, 100 μL of sample was mixed with an equal volume of 30% w/v KOH and incubated at 100°C for 10 min. Aliquots were diluted 100-fold, and two volumes of anthrone reagent (0.2% w/v anthrone in $H_2SO_4$) were

added to one volume of the diluted solution. The mixture was incubated at 40°C for 10 min, and the absorbance was then measured at 625 nm. A standard curve relating absorbance to sugar concentration was found to be linear between 20 and 100 μg sucrose per mL.

**Nitrate.** The amount of nitrate was quantified in the form of nitrite following the reduction by zinc powder, using a commercial kit for nitrate measurement (Hach, Missisauga, ON). The limit of sensitivity was reported to be around 0.2 μg nitrate per mL.

**Phosphate.** Phosphate concentration was determined by the method of Chen et al. (1956), with a sensitivity limit of about 10 μg of phosphate per mL. Briefly, one volume of ammonium molybdate solution (0.5% w/v ammonium molybdate, 2% w/v ascorbic acid in 1 M sulphuric acid) was added to an equal volume of the sample. Following incubation of the mixture at 37°C for 1 h, the absorbance was determined at 820 nm. A standard curve relating absorbance to phosphate concentration was found linear between 10 and 200 μg of phosphate per mL.

### C.6 Treatment of plant cell cultures

Cell suspension cultures grown in 250-mL Erlenmyer or 1-L nipple flasks were treated with different conditions and compounds as shown in Table 1. Triplicate samples were harvested at 48 h following such treatments. The cells were filtered through Whatman #1 filter paper using vacuum for the

determination of growth parameters, then stored at -80°C for further analysis.

**C.7 Preparation of extracts for enzyme assays.**

Frozen cells were thawed and ground in 50 mM Tris-HCl buffer (pH 7.5) containing 14 mM ß-ME and Polyclar (10% w/w). The cell homogenate was sonicated, centrifuged, and the supernatant was passed through a PD-10 column (Pharmacia) before being used as the enzyme source. Protein concentrations were then determined as described earlier.

**C.8 Flavonol sulfotransferase assay**

F-ST activity was measured according to Varin et al (1987b). Briefly, the reaction mixture contained 1 $\mu$M flavonol substrate, 0.1 $\mu$Ci [$^{35}$S]PAPS (1 $\mu$M) and up to 60 $\mu$g protein. The final volume was adjusted to 100 $\mu$L with the enzyme assay buffer (50 mM Tris-HCl, pH 7.5 containing 14mM ß-ME). The assay mixture was incubated for 15 min at 30°C, and the enzyme reaction was stopped by the addition of 20 $\mu$L of 20% v/v acetic acid, 20 $\mu$L of 0.1 M TBADP and 250 $\mu$L ethyl acetate. The sulfated reaction products were extracted in the ethyl acetate layer, and an aliquot (ca. 100 $\mu$L) was counted for radioactivity in a toluene-based scintillation fluid. The enzyme reaction product was identified by co-chromatography with reference compounds and autoradiography on X-ray film.

**C.9 Western blot analysis**

Protein extracts (ca. 20 μg) were fractionated by SDS-PAGE using 12% acrylamide gels. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes using a semi-dry electrotransfer apparatus according to the manufacturer's instructions (Bio-Rad). The blots were developed with either the anti-flavonol 3-ST immune serum (1:2000) or nonimmune serum (1:2000), as primary antibody, then with alkaline phosphatase-conjugated anti-rabbit IgG antibody as the secondary antibody.

**D. Results**

**D.1 Characterization of flavonol sulfotransferase of *F. bidentis* cell cultures.**

The crude extract and protein fractions obtained following ammonium sulfate precipitation, chromatography on PAP-agarose, and chromatofocusing on Mono P columns were subjected to F-ST enzymatic assay using quercetin, and quercetin 3-sulfate as substrates. The PAP-agarose bound fractions, which exhibited F-ST activity, were pooled and subjected to chromatofocusing on a Mono P column. Figure 2A shows the Mono P fractions which exhibited F3-ST activity utilizing quercetin, but not quercetin 3-sulfate, as the substrate giving rise to quercetin 3-sulfate as the enzyme reaction product. Therefore they were considered to have the

F3-ST activity (Fig. 2B). The fraction with highest enzymatic activity exhibited a pI of 5.4. The active protein fractions were then subjected to western blot analysis using a polyclonal antibody raised against F. chloraefolia F3-ST. The antibody revealed that the F. bidentis F3-ST migrated at an estimated molecular weight of 35 kD (Fig. 2C), as compared with the prestained protein molecular weight standards.

**D.2 Nutrient depletion by F. bidentis cell cultures**

The relative changes, calculated as averages of triplicate samples, in the amounts of sucrose, ammonium, nitrate and phosphate of MS and B5 culture media are shown in Fig. 3A and 3B, respectively. The contents of sucrose and phosphate in both media declined in the same manner; ca. 40% was left in the media on day 4, and less than 10% remained on day 10 of culture growth. Whereas the levels of nitrate and ammonium in the MS medium remained constant during the 10-day growth period, those of B5 steadily declined to their lowest level by day 18. Although, the growth rates of cells cultured in both media followed a sigmoidal curve with the F3-ST enzymatic activity reaching a maximum 2 days after subculture, the enzyme activity in B5 cultures was ca 15% lower than that of MS cultures (Fig. 3C and 3D). The pH of both media remained relatively constant (5.4 ± 0.2) throughout the growth period.

**D.3 Treatment of cell cultures with nutrients, phytohormones, elicitors and flavonols.**

To test the hypothesis that flavonol sulfates might be salt stress metabolites (Harborne, 1975), the cultures were grown in media containing various concentrations of the major ionsin combination; including ammonium, nitrate, phosphate, and sulfate. However, no significant change in either growth or F3-ST activity was observed following two days of treatment (Table 2).

To study the effects of phytohormones, the control cultures were subcultured into conditioned media. The latter contained various concentrations of 2,4-D and kinetin. Of the different treatments used, only the synthetic auxin, 2,4-D showed an activation of F3-ST activity. The enzyme activity was increased by ca. 2-fold in the conditioned media containing 25 $\mu$M 2,4-D and 0.45 $\mu$M kinetin, as compared with the control (Table. 2). There were no significant effects of kinetin, whether added in the presence or absence of 2,4-D, on either growth or F3-ST activity.

F. bidentis cultures were treated with elicitors which have previously been reported to affect flavonoid biosynthesis (for review see Hahlbrock et al., 1980; Dixon et al., 1986). We observed that abiotic elicitors at low concentrations (e.g. 0.05 mM cupric chloride and mercuric chloride) were extremely harmful to F. bidentis cell cultures. Following 6 h of exposures to the elicitors, less than 50 % of cells remained

viable. Of the biotic elicitors used, pectinase (0.2% w/v) slightly induced the growth rate of the cell cultures, but with no effect on F3-ST activity. Other biotic elicitors (cellulase, *Pythium* and *Botrytis* lysates) showed no significant effect either on culture growth or F3-ST activity.

The results of treatments with quercetin and quercetin 3-sulfate, the flavonol substrate and enzyme product of F3-ST respectively, were studied. No significant effect was observed in the cultures treated with 3 μM quercetin, whereas a decline in F3-ST activity was observed in the cultures treated with 3 μM quercetin 3-sulfate. F3-ST activity decreased to ca 40% of that of control cultures, 2 days following their subculture into the conditioned media.

**E. Discussion**

Intact leaves of F. bidentis accumulate a variety of flavonol mono- to tetrasulfates, suggesting the possibility of the presence of a family of F-STs involved in their biosynthesis (Varin 1992). In contrast with the intact tissues, F. bidentis cell suspension cultures exhibit very low levels of F-ST activity and do not accumulate any flavonol sulfate esters (Bleichert et al., 1989). We have demonstrated the presence of F3-ST enzymatic activity in cell suspension cultures. Although the F7-ST of F. bidentis has previously been purified from terminal buds (Varin and Ibrahim, 1991), no extractable F7-ST activity was found in the cell cultures.

Because the cell cultures were originally established from terminal bud explants, we anticipated to obtain some extractable F-ST activities, other than the F3-ST. The differences in the extractable F-ST activities and the levels of flavonol sulfates from intact and cultured tissues suggest the existence of a mechanism regulating the levels of flavonol sulfates and the expression of their biosynthetic enzymes that is controlled by environmental and/or developmental factors.

The nutrient requirements of F. bidentis cell cultures were determined in two standard culture media, MS and B5, containing 3% w/v sucrose and the same levels of phytohormones (4.5 $\mu$M 2,4-D and 0.45 $\mu$M kinetin). MS culture medium contains much higher levels of nitrate, potassium and ammonium ions than the B5 medium. F. bidentis cultures exhibited similar growth rates in both media, although the amount of both nitrate and ammonium ions were depleted fairly rapidly in the B5 cultures. Sucrose and phosphate appeared to be the limiting factors in both nutrient media (Fig. 3A and 3B). However, when F. bidentis cell cultures were grown in higher concentrations of sucrose (up to 7% w/v) and phosphate (up to 5 mM), only slightly higher growth rates were observed (Table 2).

The accumulation of flavonol sulfates by Flaveria spp., which naturally grows in marshy habitat, has previously been ascribed to salt tolerance by flavonoid sulfate-accumulating plants (Harborne, 1975). However, the treatment of F.

bidentis cell cultures with high concentration of ions, in the form of ammonium nitrate, potassium nitrate, potassium phosphate, sodium phosphate, calcium chloride, or magnesium sulfate, either alone or in a combination with other components, did not seem to affect F3-ST enzymatic activity. Thus, F-ST and flavonol sulfates of F. bidentis are unlikely the products of a response to salt stress, at least in cell cultures.

Previous reports have shown that phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), catalyzing the early steps of the phenylpropanoid and flavonoid biosynthetic pathways, respectively, are involved in stress responses, being induced in alfalfa cell cultures upon treatment with elicitors (Jorin and Di: ,n, ,990; Junghans et al., 1993). However, F3-ST catalyses a later step in sulfated flavonoid biosynthesis, and is not affected by either the biotic or abiotic elicitors tested. These results suggest that F3-ST is not directly involved in a stress response.

We found that the F3-ST activity of F. bidentis cell cultures was activated by 2,4-D treatment. Although the mechanism by which 2,4-D acts at the cellular level is not well understood, this synthetic auxin is commonly used to study similar physiological effects, *e.g.* down regulation of PAL and CHS in carrot cell cultures (Ozeki et al., 1990). The 2,4-D activation of F3-ST, which catalyses a later step in flavonoid biosynthesis, could well be important for the

regulatory role of flavonol sulfates in the auxin polar transport. In addition, the finding that quercetin 3-sulfate, the F3-ST enzyme product, reduced the F3-ST activity, suggests that a feedback inhibition may take place. PAL and CHS have been reported to be regulated by the biosynthetic pathway intermediates, *trans*-cinnamic acid, *trans*-4-coumaric acid (Orr et al., 1993; Loake et al., 1991). Although the accumulation of flavonol sulfates in terminal buds of F. bidentis has previously been reported to be ca 30 μM (Hannoufa et al., 1991), the accumulation of these metabolites in cell cultures could not be detected. At this stage we can not discern how the external application of quercetin 3-sulfate affects an internal pool of flavonol conjugates, and whether the change in the flavonol sulfate level or product-uptake gives rise to F3-ST inhibition. Therefore, a study at the molecular level of F3-ST gene expression in response to flavonol sulfate levels would provide more information on this regulation. Although the mechanism underlying the F3-ST regulation is not clear, the finding that quercetin 3-sulfate exhibits an inhibitory effect on its biosynthetic enzyme suggests a control by an internal level of flavonol sulfates.

## F. Literature Cited

Barron D, Varin L, Ibrahim RK (1986) Identification and biosynthesis of glucosylated and sulfated flavonols in Flaveria bidentis. Z. Naturforsch. 41c: 813-819

Bleichert E, Varin L, Hannoufa A, Ibrahim RK (1989) In vitro culture of Flaveria spp. and its potential for the biosynthesis of sulfated flavonoids. Plant Physiol 89: S-187

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72: 248-254

Chapple CCS, Vogt T, Ellis B, Somerville CR (1992) An Arabidopsis mutant defective in the general phenylpropanoid pathway. Plant Cell 4: 1413-1424

Chen PS, Toribara TY, Warner M (1956) Microdetermination of phosphorus. Anal Chem 28: 1756-1758

Dixon RA (1986) The phytoalexin response: elicitation, signalling and control of host gene expression. Biol Rev 61: 239-291

Dixon RA, Dey PM, Lamb CJ (1983) Phytoalexins: enzymology and molecular biology. Adv Enzymol Relat Areas Mol Biol 55: 1-135

Djordjevic MA, Redmond JW, Batley M, Rolfe BG (1987) Clovers secret compounds which either stimulate or repress nod gene function in Rhizobium trifolii. EMBO J 6: 1173-1179

Edwards R, Dixon R (1991) Isoflavone O-methyltransferase activities in elicitor-treated cell suspension cultures of Medicago sativa. Phytochemistry 30: 2597-2606

Faulkner IJ, Rubery PH (1992) Flavonoids and flavonoid sulfates as probes of auxin-transport regulation in Cucurbita pepo hypocotyl segments and vesicles. Planta 186: 618-625

Firmin JL, Wilson KE, Rossen L, Johnson AWB (1986) Flavonoid activation of nodulation genes in Rhizobium reversed by other compounds present in plants. Nature 324: 90-92

Gamborg OL, Wetter LR (1975) Plant Tissue Culture Methods. NRC Publication, Ottawa

Handel EV (1968) Direct microdetermination of sucrose. Anal Biochem 22: 280-283

Hannoufa A, Varin L, Ibrahim RK (1991) Spatial distribution of flavonoid conjugates and their enzymes in Flaveria bidentis. Plant Physiol 97: 259-263

Harborne JB (1975) Flavonoid sulphates: A new class of natural product of ecological significance in plants. In L Reinhold, JB Harborne, eds, Progress in Phytochemistry, Vol 4. Pergamon Press, New York, pp 189-208

Jacobs M, Rubery PH (1988) Naturally occurring auxin transport regulators. Science 241: 346-349

Jorin J, Dixon RA (1990) Stress responses in alfalfa (Medicago sativa) II. Purification, characterization and induction of phenylalanine ammonia-lyase isoform from elicitor-treated cell suspension cultures. Plant Physiol 92: 447-455

Junghans H, Dalkin K, Dixon RA (1993) Stress responses in alfalfa (Medicago sativa) XV Characterization and expression patterns of members of a subset of the chalcone synthase multigene family. Plant Mol Biol 22: 239-253

Kosslak RM, Bookland R, Barkei J, Paaren HE, Appelbaum ER (1987) Induction of Bradyrhizobium japonicum common nod genes by isoflavones isolated from Glycine max. Proc Natl Acad Sci USA 84: 7428-7432

Lamb CJ, Lawton MA, Dron M, Dixon RA (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56: 215-224

Li J, Ou-bee TM, Raba R, Amundson RG, Last RL (1993) Arabidopsis flavonoid mutants are hypersensitive to UV-B radiation. Plant Cell 5: 171-179

Long S (1989) Rhizobium-legume nodulation: life together in the underground. Cell 56: 203-214

Loake GJ, Choudhary AD, Harrison MJ, Mavandad M, Lamb CJ, Dixon RA (1991) Phenylpropanoid pathway intermediates regulate transient expression of a chalcone synthase gene promoter. Plant Cell 3: 829-840

Lynn DG, Chang M (1990) Phenolic signal in cohabitation: implications for plant development. Annu Rev Plant Physiol 41: 497-526

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473-497

Orr JD, Edwards R, Dixon RA (1993) Stress responses in alfalfa (Medicago sativa L.) XIV. Changes in the levels of phenylpropanoid pathway intermediates in relation to regulation of L-phenylalanine ammonia-lyase in elicitor-treated cell suspension cultures. Plant Physiol 101: 847-856

Ozeki Y, Komamine A, Tanaka Y (1990) Induction and repression of phenylalanine ammonia-lyase and chalcone synthase enzyme proteins and mRNAs in carrot cell suspension cultures regulated by 2,4-D. Physiol Plant 78: 400-408

Pollak PE, Vogt T, Mo Y, Taylor L (1993) Chalcone synthase and flavonol accumulation in stigma and anthers of Petunia hybrida. Plant Physiol 102: 925-932

Redmond JW, Batley M, Djordjevic MA, Innes RW, Kuempel PL, Rolfe BG (1986) Flavones induce expression of nodulation genes in Rhizobium. Nature 323: 632-634

Steward FC, Caplin SM, Millar FK (1952) Investigations on growth and metabolism of plant cells: I. New techniques for the investigation of metabolism, nutrition and growth in undifferentiated cells. Ann Bot 16: 57-77

van der Meer IM, Stam M, van Tunen AJ, Mol JNM, Stuitje AR (1992) Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. Plant Cell 4: 253-262

Varin L (1992) Flavonoid sulfation: phytochemistry, enzymology and molecular biology. Rec Adv Phytochem 26: 233-252

Varin L, Ibrahim RK (1989) Partial purification and characterization of three flavonol-specific sulfotransferases from Flaveria chloraefolia. Plant Physiol 90: 977-981

Varin L, Ibrahim RK (1991) Partial purification and some properties of flavonol 7-sulfotransferase from Flaveria bidentis. Plant Physiol 95:1254-1258

Varin L, Ibrahim RK (1992) Novel flavonol 3-sulfotransferase: purification, kinetic properties, and amino acid sequence. J Biol Chem 267: 1858-1863

Varin L, Barron D, Ibrahim RK (1987a) Enzymatic synthesis of sulphated flavonols in Flaveria. Phytochemistry 26: 135-138

Varin L, Barron D, Ibrahim RK (1987b) Enzymatic assay for flavonoid sulfotransferase. Anal Biochem **161**: 176-180

Vogt T, Pollak P, Tarlyn N, Taylor LP (1994) Pollination- or wounding kaempferol accumulation in petunia stigma enhance seed production. Plant Cell **6**: 11-23

**Table 1.** Treatments of *F. bidentis* tissue culture using different conditions and compounds.

| Condition | Compound | Concentration |
|---|---|---|
| **Nutrient medium** | | |
| **MS medium**[a] | Diluted MS | 0.25x, 0.5x |
| | Concentrated | 2x, 4x |
| **Modified MS medium**[b] | | |
| | Ammonium ($NH_4^+$) | 41.2, 82.4 mM |
| | Nitrate ($NO_3^-$) | 78.8, 157.6 mM |
| | Phosphate ($PO_4^{3-}$) | 2.5, 5.0 mM |
| | Sulfate ($SO_4^{2-}$) | 6.0, 12.0 mM |
| **Elicitors** | | |
| **Abiotic elicitors** | | |
| | $CuCl_2$ | 0.05 to 1.0 mM |
| | $HgCl_2$ | 0.05 to 0.5 mM |
| **Biotic elicitors** | | |
| | Cellulase | 0.05 to 1.5 % w/v |
| | Pectinase | 0.05 to 1.5 % w/v |
| | *Pythium* lysate | 0.5 to 3.0 % v/v |
| | *Botrytis* lysate | 0.5 to 3.0 % v/v |
| **Phytohormones** | 2,4-D | 1.0 to 200.0 μM |
| | Kinetin | 0.1 to 100.0 μM |
| **Flavonols** | Quercetin | 1.0 to 50.0 μM |
| | Quercetin 3-sulfate | 1.0 and 5.0 μM |

[a] MS medium contains 20.6 mM $NH_4^+$, 39.4 mM $NO_3^-$, 1.25 mM $PO_4^{3-}$, and 3.0 mM $SO_4^{2-}$.

[b] Modified MS medium contains various concentrations of the major ions: $NH_4^+$, $NO_3^-$, $PO_4^{3-}$, and $SO_4^{2-}$.

**Table 2.** Fresh weight, cell viability, and flavonol 3-sulfotransferase activity of F. bidentis cell cultures observed under different growth conditions.

| Conditions | Fresh weight (mg/10ml) | %cell viability | F3-ST ($10^{-3}$ pKat/mg protein) |
|---|---|---|---|
| Control[1] | 0.65 ± 0.05[3] | 100 | 253 ± 20 |
| Diluted MS | | | |
| 0.25x | 0.66 ± 0.06 | 100 | 250 ± 15 |
| 0.5x | 0.65 ± 0.02 | 95 | 260 ± 10 |
| Concentrated MS | | | |
| 2x | 0.70 ± 0.07 | 97 | 230 ± 20 |
| 4x | 0.60 ± 0.04 | 98 | 250 ± 35 |
| Modified MS medium | | | |
| Sucrose (w/v) | | | |
| 5% | 0.80 ± 0.09 | 94 | 270 ± 20 |
| 7% | 0.70 ± 0.03 | 96 | 257 ± 15 |
| Ammonium (mM) | | | |
| 41.2 | 0.60 ± 0.03 | 93 | 240 ± 13 |
| 82.4 | 0.55 ± 0.05 | 90 | 250 ± 20 |
| Phosphate (mM) | | | |
| 2.5 | 0.70 ± 0.05 | 98 | 240 ± 20 |
| 5.0 | 0.50 ± 0.1 | 90 | 270 ± 30 |
| Elicitors[2] | | | |
| Abiotic elicitors | | | |
| $CuCl_2$ (0.05 mM) | 0.30 ± 0.07 | 50 | 100 ± 50 |
| $HgCl_2$ (0.05 mM) | 0.25 ± 0.09 | 48 | 270 ± 100 |
| Biotic elicitors | | | |
| Cellulase (0.1% w/v) | 0.60 ± 0.1 | 89 | 260 ± 35 |
| Pectinase (0.2% w/v) | 0.70 ± 0.09 | 90 | 240 ± 20 |
| Pythium lysate (1% v/v) | 0.50 ± 0.09 | 78 | 200 ± 50 |
| Botrytis lysate (1% v/v) | 0.60 ± 0.08 | 80 | 200 ± 60 |
| Phytohormones[2] | | | |
| 2,4-D (25 μM) | 0.70 ± 0.05 | 98 | 400 ± 60 |
| kinetin (3 μM) | 0.65 ± 0.1 | 97 | 250 ± 30 |
| Flavonols[2] | | | |
| Quercetin (Q, 3 μM) | 0.65 ± 0.04 | 95 | 250 ± 30 |
| Q 3-sulfate (3 μM) | 0.60 ± 0.03 | 93 | 100 ± 20 |

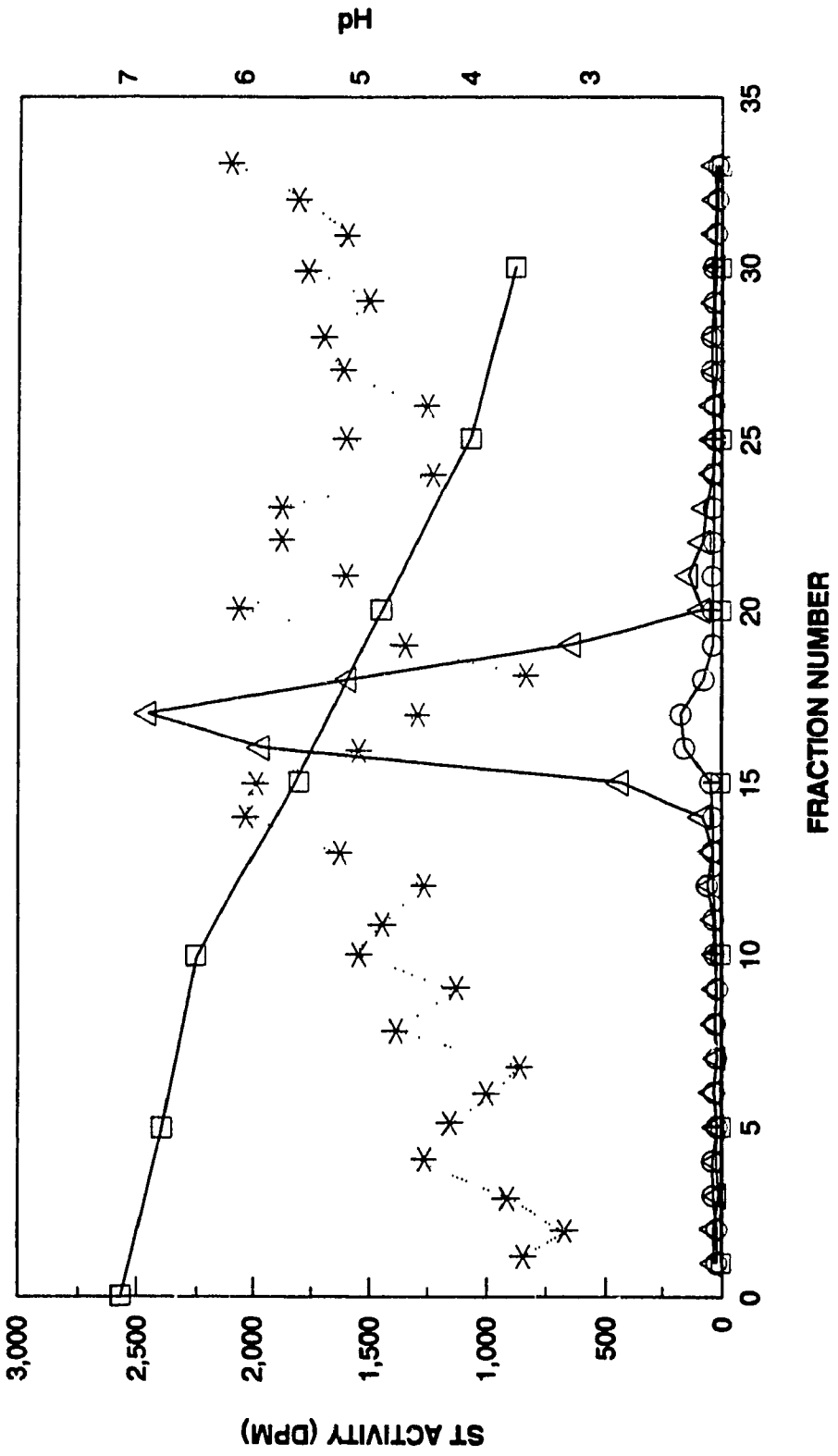
[1] Control F. bidentis cell cultures were grown in MS medium.

[2] Dose response curves were obtained using various concentrations as listed in Table 1. The optimum concentration of each compound was used to obtain fresh weight, cell viability, and flavonol sulfotransferase activity values. The latter were averages of triplicate samples.

[3] Standard error of mean.

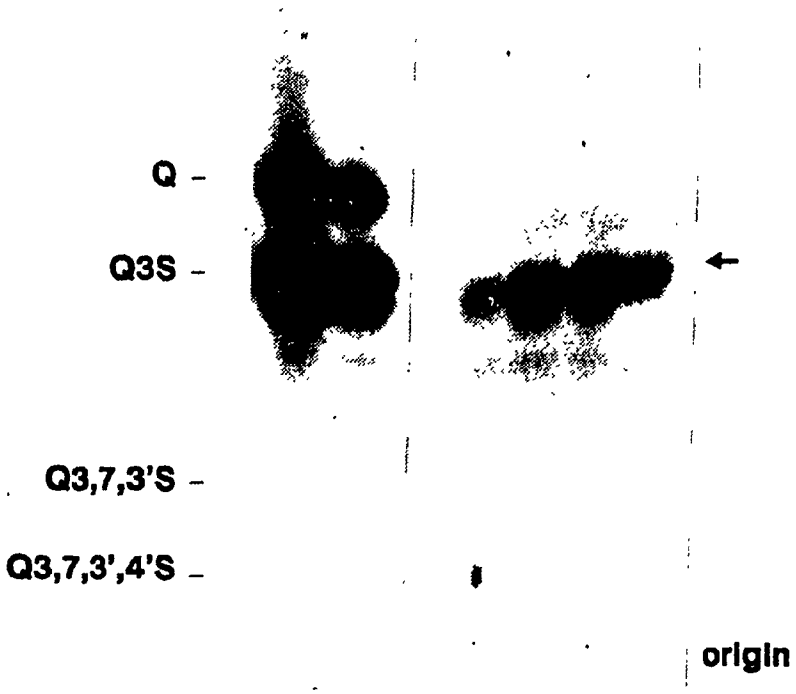
**Figure 1.** Three-day-old F. bidentis cell suspension cultures grown in MS medium. The bar indicates a relative size of 12.5 μm.

**Figure 2.** A) Elution profile of the F-ST activity after chromatofocusing on Mono P. Protein fractions (ca 1-mL) were eluted with Polybuffer 74 which generated a pH gradient between pH 7 and 4. The enzyme assays were carried out using quercetin (-Δ-) and quercetin 3-sulfate (-O-) as substrates. The protein contents of the eluted fractions (-*-) were monitored by absorbance at 280 nm. (B) Autoradiogram of the chromatographed enzyme reaction products from crude extract (ex), bound PAP agarose protein fraction (pap) and Mono P protein fraction no. 15 to 18, after F-ST assay using quercetin as substrate. The relative migration of reference compounds are marked on the left: Q, quercetin; Q3S, quercetin 3-sulfate, Q3,7,3',4'S, quercetin 3,7,3',4'-tetrasulfate. (C) Western blot analysis of the protein fractions. The samples were loaded with crude protein extract (lane a), bound PAP-agarose protein fraction (lane b), and Mono P-purified F3-ST fractions no. 15, 16, 17 and 18 (lanes c to f). The F3-ST of F. bidentis was detected by a polyclonal antibody raised against F. chloraefolia F3-ST, and migrated with an estimated molecular weight of 35 kD.


pH
7
6
5
4
3
ST ACTIVITY (DPM)
3,000
2,500
2,000
1,500
1,000
500
0
FRACTION NUMBER
0
5
10
15
20
25
30
35

*24*

*2B*



*2C*

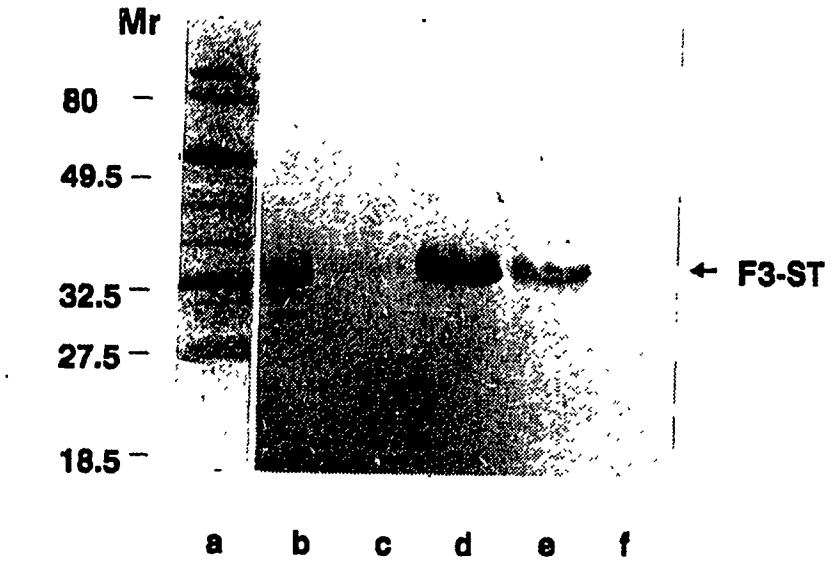
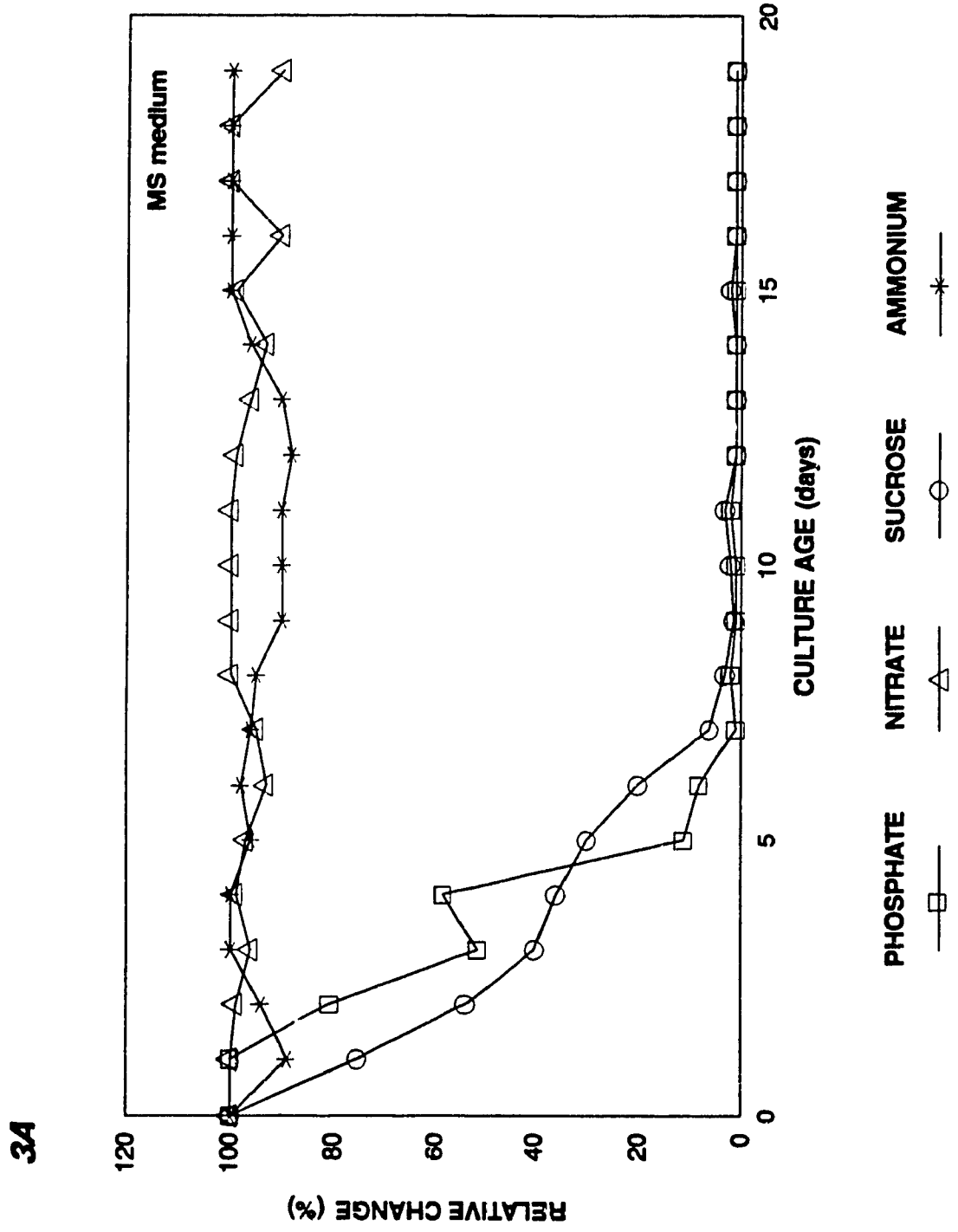
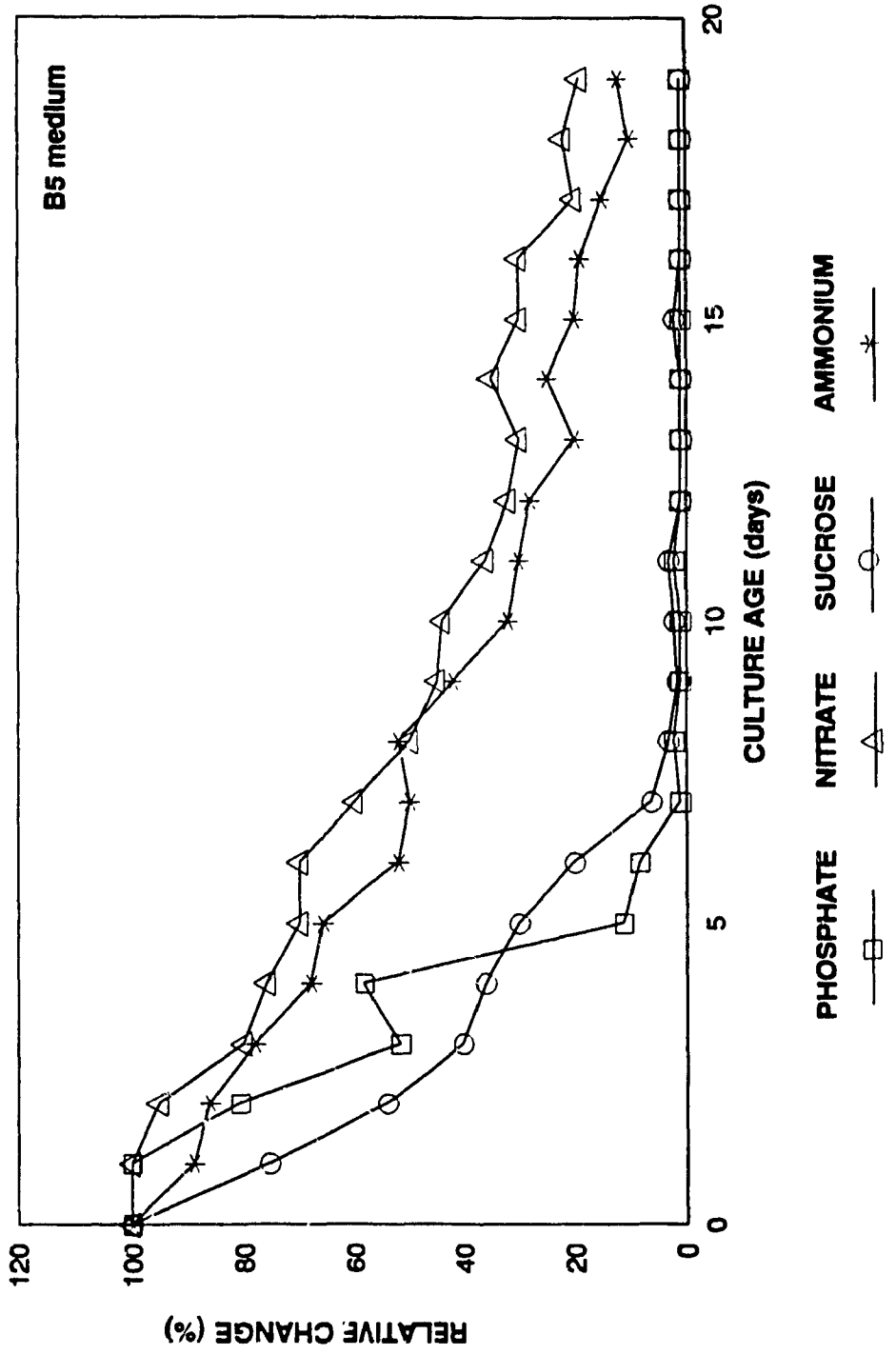
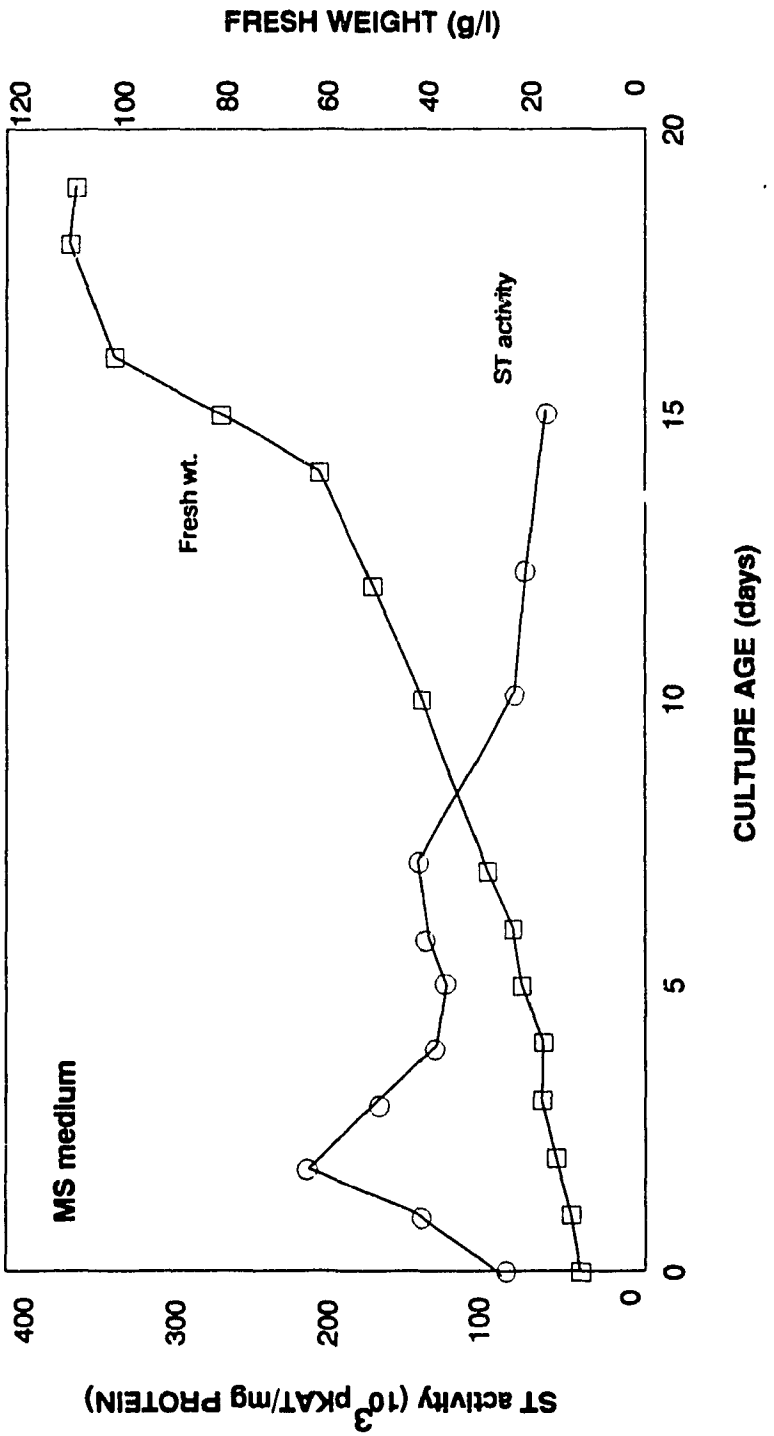


**Figure 3.** Depletion of the major nutrients in MS medium (A) and those of B5 media (B) during growth of F. bidentis cell suspension cultures. The relative changes (%) of sucrose (-○-), ammonium (-*-), nitrate (-△-), and phosphate (-□-) are the averages of triplicate sample determinations. Panels C and D show the growth and F3-ST activity that were monitored during culture growth in MS and B5 media, respectively.

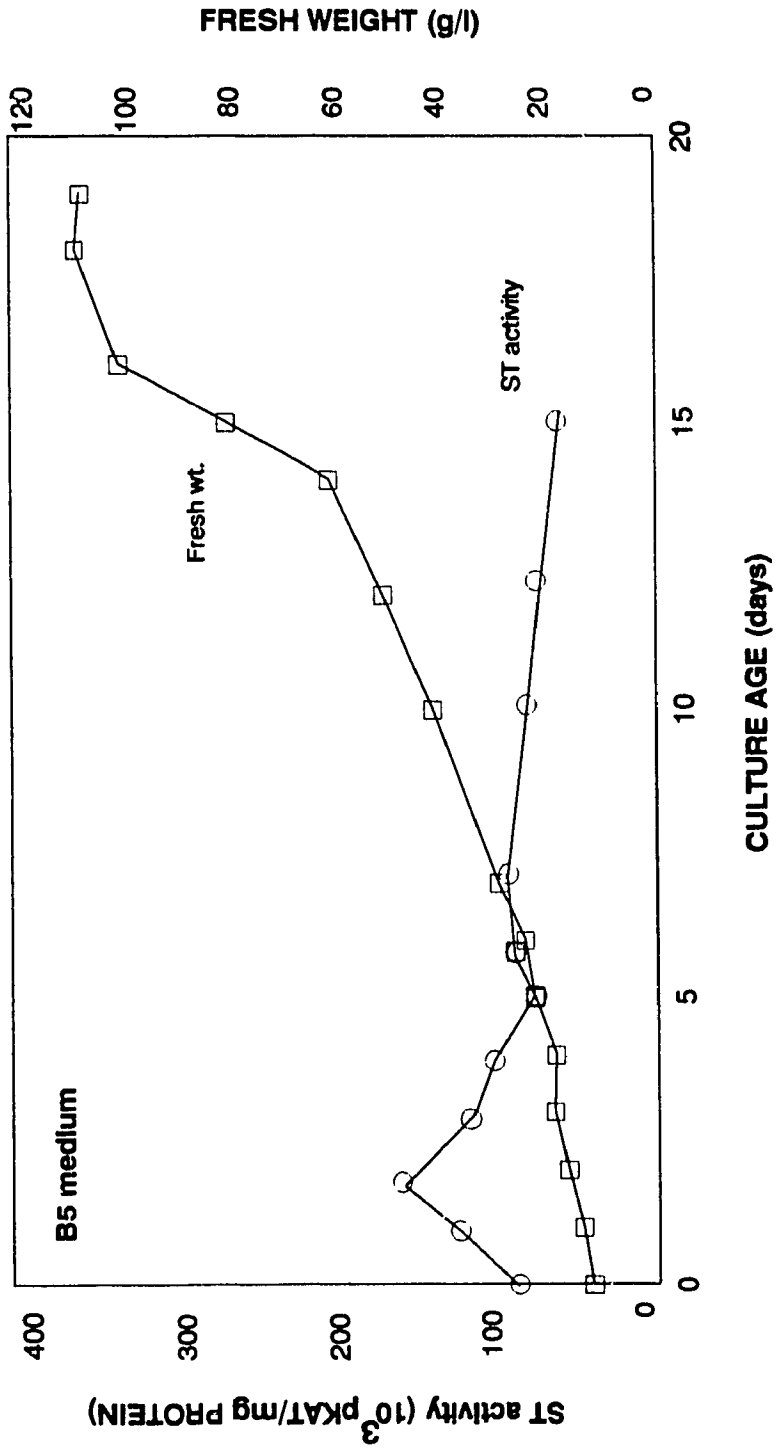

34
MS medium
RELATIVE CHANGE (%)
120
100
80
60
40
20
0
0
5
10
15
20
CULTURE AGE (days)
PHOSPHATE
NITRATE
SUCROSE
AMMONIUM


B5 medium
RELATIVE CHANGE (%)
120
100
80
60
40
20
0
0
5
10
15
20
CULTURE AGE (days)
PHOSPHATE
NITRATE
SUCROSE
AMMONIUM

3B


FRESH WEIGHT (g/l)
120
100
80
60
40
20
0
ST activity
Fresh wt.
MS medium
400
300
200
100
0
ST activity ($10^3$ pKAT/mg PROTEIN)
0
5
10
15
20
CULTURE AGE (days)

3C


FRESH WEIGHT (g/l)
120
100
80
60
40
20
0
20
15
10
5
0
CULTURE AGE (days)
ST activity
Fresh wt.
B5 medium
400
300
200
100
0
ST activity (10³ pKAT/mg PROTEIN)

3D

## CHAPTER III

# Cloning and Regulation of Flavonol 3-Sulfotransferase in Cell Suspension Cultures of *Flaveria bidentis*

## A. Abstract

*Flaveria spp.* accumulate flavonol sulfate esters whose biosynthesis is catalysed by a number of position-specific flavonol sulfotransferases. Although the accumulation of sulfated flavonols appears to be tissue-specific, developmentally-regulated, and to vary among related species, little is known about the mechanism of regulation controlling the synthesis of these metabolites. In the present work, we report the isolation of a cDNA clone from *F. bidentis* (pBFST3) encoding flavonol 3-sulfotransferase (F3-ST) which catalyses the first step in the biosynthesis of flavonol polysulfates. This clone (pBFST3) was expressed in *E. coli* and produced a F3-ST with high affinity for the flavonol aglycones, quercetin and its 7-methyl derivative, rhamnetin. In addition, the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) was shown to induce F3-ST enzyme activity and F3-ST mRNA transcript levels in cell cultures of *F. bidentis*. The F3-ST mRNA levels increased within the first 3 hours, reaching a maximum after 24 hours of treatment and remained elevated up to 48 hours. Treatments with either quercetin 3-sulfate or quercetin 3,7,4'-trisulfate reduced F3-ST enzyme activity in

cell cultures, but had no effect on the transcript levels. These results are discussed in relation to the putative role of flavonoid conjugates in the regulation of auxin transport.

**B. Introduction**

Flavonoid sulfates are of common occurrence in plants (Harborne, 1975), especially in the Asteraceae. The most commonly found compounds are mono- and disulfate esters of flavones and flavonols or their methyl ethers and, less commonly, of their glycosylated derivatives. Tri- and tetrasulfated esters have also been identified, although these compounds have a more limited taxonomic distribution (Barron et al., 1988). Recently, a number of position-specific flavonol sulfotransferases (F-STs) have been characterized from Flaveria chloraefolia (Varin and Ibrahim, 1989) and F. bidentis (Varin and Ibrahim, 1991). Different enzymes exhibited strict specificity for positions 3 of flavonol aglycones (F3-ST), 3' or 4' of flavonol 3-sulfate (F3'/F4'-ST) and 7 of flavonol 3,3'- or 3,4'-disulfates (F7-ST), thus establishing an enzymatic sequence for the formation of flavonol polysulfates (Varin, 1992).

F. bidentis (Asteraceae) accumulates flavonol mono- to tetrasulfate esters (Barron et al., 1986) and exhibits several ST activities (Varin et al., 1987a). F3-ST activity of F. bidentis has recently been shown to be regulated with respect to plant development, being highest in the shoot tips and the

first pair of expanded leaves and lowest in mature leaves (Hannoufa et al., 1991). In contrast, cell suspension cultures of F. bidentis exhibit very low constitutive levels of F3-ST activity and do not accumulate any flavonol sulfate (Bleichert et al., 1989).

Studies by Jacobs and Rubery (1988) showed that exogenous treatments of etiolated Curcubita pepo hypocotyls with either quercetin or rhamnetin inhibit auxin transport in tissue sections. Transport is inhibited by a decreased auxin efflux at the basal end of the stem cells and is mediated by competitive binding of quercetin and rhamnetin to a membrane protein, the naphthylphthalamic acid (NPA) receptor. It was later shown that sulfated esters of quercetin are antagonists of quercetin in affecting auxin transport in the microsomal preparations of C. pepo (Faulkner and Rubery, 1992). Quercetin 3-sulfate has also been shown to bind to the NPA receptor and de-repress the auxin efflux inhibition caused by quercetin. Because flavonol 3-sulfate is a naturally occurring compound in F. bidentis and F3-ST is known to be spatially and developmentally regulated in this species (Hannoufa et al., 1991), the effects of the synthetic auxin, 2,4-D, on the regulation of F3-ST activity and gene expression in cell suspension cultures of F. bidentis were investigated.

In this study, we report the isolation of a cDNA clone encoding F3-ST in F. bidentis. This clone was used to demonstrate that steady state mRNA levels of the gene encoding

F3-ST are regulated by 2,4-D. Neither of the sulfate esters, quercetin 3-sulfate or quercetin 3,7,4'-trisulfate, had any effect on the steady state mRNA levels of the F3-ST.

## C. Materials and Methods

### C.1 Plant material

Seeds of Flaveria bidentis var. angustifolia O.K. (Asteraceae) were kindly provided by Dr. H.R. Juliani, University of Cordoba, Argentina. Seeds were germinated in vermiculite on top of potting soil, and plants were further propagated by cuttings.

A callus culture of F. bidentis was initiated from leaf disks and maintained on an MS semi-solid medium containing 3% w/v sucrose, 4.5 $\mu$M 2,4-D and 0.45 $\mu$M kinetin (Bleichert et al., 1989). Calli were broken into small pieces and transferred to a liquid medium of the same composition. The suspension culture was maintained in the light, at room temperature, either in Erlenmeyer flasks agitated on an orbital shaker at 110 rpm, or in 1-L nipple flasks rotating centripetally at 4 rpm.

### C.2 Chemicals

2,4-D was purchased from Sigma (St. Louis, MO). 3'-Phosphoadenosine 5'-phospho-[$^{35}$S]sulfate (PAPS, 1.57 Ci/mmol) was purchased from New England Nuclear (Boston, MA), and

quercetin 3-sulfate from Sarsynthèse (Merignac, France). Tetrabutylammonium dihydrogen phosphate (TBADP) was obtained from Aldrich Chemical Co. (Milwaukee, WI), and the immunodetection kit from Bio-Rad (Mississauga, ON). UniZap cDNA synthesis kit was obtained from Stratagene (La Jolla, CA) and Sequenase version I sequencing kit was from USB (Cleveland, OH). All other chemicals were of analytical grade.

**C.3 cDNA cloning of F3-ST**

Total RNA was isolated from the shoot tips of F. bidentis (Logemann et al., 1987) and poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972). A cDNA library was constructed in lambda UniZap II according to the manufacturer's instructions (Stratagene). Approximately $6.0 \times 10^5$ plaques were screened using polyclonal antibodies that were raised against the F3-ST of F. chloraefolia (Varin and Ibrahim, 1992). The plasmid was rescued from positive clones by in vivo excision according to the manufacturer's instructions (Stratagene). Clones were further screened by western blot analysis of E. coli lysates from positive clones, and subsequently by screening for F3-ST activity as described below. A single clone, pBFST3, which expressed F3-ST activity was chosen for DNA sequencing. A series of nested deletions of this clone were created using Exonuclease III and Mung bean nuclease (Sambrook et al.,

1989). DNA sequences of both strands were determined by the dideoxy chain termination method (Sanger et al., 1977).

**C.4 Treatment of plant cell cultures**

Ten-day old cell suspension cultures were treated with varying concentrations of 2,4-D, or with the flavonols quercetin, quercetin 3-sulfate or quercetin 3,7,4'-trisulfate under sterile conditions. Triplicate samples were harvested at the indicated time intervals by filtration through Whatman #1 filter paper and rinsed extensively with MS medium to remove the residual 2,4-D and flavonols. The harvested cells were weighed, frozen in liquid nitrogen, and then stored at -70°C.

To determine the induction kinetics of F3-ST activity by 2,4-D, 10-d old nipple-flask cultures were exposed to 25 $\mu$M 2,4-D and 20-mL samples were taken at 3-h intervals and used for the determination of F3-ST activity. Additional experiments were conducted using actinomycin D, which was added to the Erlenmyer flask cultures at 0.1 mM final concentration, 3 h prior to treatment with 2,4-D. Control experiments were performed in which 2,4-D was substituted by water. The effect of flavonols on F3-ST activity was determined by transferring 25 mL of 10-d old cell suspension cultures to 250 mL of MS medium containing either quercetin, quercetin 3-sulfate, or quercetin 3,7,4'-trisulfate at a final concentration of 3 $\mu$M. Samples were taken daily and assayed

for F-ST activity.

**C.5 Preparation of protein extracts**

**E. coli lysates** One hundred and fifty μL of overnight bacterial cultures, grown in LB culture medium with tetracycline (100 $\mu g.mL^{-1}$) and ampicillin (50 $\mu g.mL^{-1}$), were used to inoculate 3 mL of the same medium. IPTG was added after 1 h to a final concentration of 1 mM. After 2 h, the culture was centrifuged (10,000xg), and the cell pellets were resuspended in one mL of the enzyme assay buffer (50 mM Tris-HCl, pH 7.5, 14 mM 2-ME), then lysed by sonication. The lysate was cleared by centrifugation (10,000xg) and the supernatant was desalted by passage through a PD-10 column (Pharmacia). The desalted protein preparations were used as the enzyme source for ST assay.

**Cell-free extracts from cell cultures** Frozen tissues were thawed and ground in ca 2 vol of 50 mM Tris-HCl buffer (pH 7.5) containing 14 mM 2-ME and Polyclar (Polyclar:cells, 1:10, w/w). The homogenate was sonicated, centrifuged, and the supernatant was desalted by passage through a PD-10 column (Pharmacia), before being used as the enzyme source.

**C.6 Sulfotransferase assay**

Flavonol ST activity was measured according to Varin et al. (1987b). The reaction mixture contained 1 μM of the flavonol substrate, 1 μM [$^{35}$S]PAPS (0.1 μCi) and up to 60 μg

protein in a total volume of 100 $\mu$L. The sulfated reaction product was extracted in 0.1% w/v TBADP-ethyl acetate and counted for radioactivity in a toluene-based scintillation fluid. It was identified by co-chromatography with reference compounds and autoradiography on X-ray film.

**C.7 Protein determination**

Protein concentrations were determined according to the method of Bradford (1976) using the Bio-Rad reagent and BSA as the protein standard.

**C.8 Northern blot analysis**

Total RNA was isolated according to Logemann et al. (1987) and quantitated by UV spectrophotometry. Equal amounts (5 or 10 $\mu$g) of total RNA were fractionated on a 1% agarose formaldehyde gel. The gel was then stained with ethidium bromide and blotted onto Hybond N membrane (Amersham, IL). Prehybridizations and hybridizations were performed as described by Gulick and Dvorak (1990). The F3-ST probe was synthesized with the random primer labelling method. The blots were then washed 3 times with a mixture containing 0.1 x SSC, 0.1% SDS and 0.1% sodium pyrophosphate, for 20 min at 55°C. Equal sample loading was confirmed by probing blots with a ribosomal RNA control probe. The blots were then exposed to Kodak XAR film with an intensifying screen at -80°C.

**C.9 Western blot analysis**

**E. coli lysates.** Protein extracts (20 μg) were fractionated by SDS-PAGE using 12% acrylamide gels. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes using a semi-dry electrotransfer apparatus according to the manufacturer's instructions (Bio-Rad). The blot was developed with anti-F3-ST immune serum (1:2000) and nonimmune serum (1:2000) as primary antibody, then with alkaline phosphatase-conjugated anti-rabbit IgG antibody as the secondary antibody.

**Cell-free extracts from cell cultures.** Samples were ground in liquid nitrogen to a fine powder, and extracted with SDS sample buffer (1:2, w/v). About 20 μg of protein extracts were fractionated under the same conditions as described above. Equal sample loading was confirmed by Coomassie blue staining. The blot was developed with anti-F3-ST immune serum (1:500).

**D. Results**

**D.1 Characterization of F3-ST cDNA clone**

A clone, designated as pBFST3, was identified by antibody screening of a F. bidentis cDNA library and subsequent western blot analysis of E. coli lysate of a strain carrying the plasmid. The antibody reacted with a protein band from the lysate with an estimated molecular weight of 37 kD (Fig. 1A,

lane b); this is ca. 5 kDa smaller than the predicted molecular weight of the cloned gene product, which includes 59 amino acid residues encoded by the vector and the 5'-untranslated region of the cDNA. The identity of the clone, as the F3-ST, was further confirmed by measuring the F3-ST activity in the E. coli lysate. The level of F3-ST activity in E. coli transformed with pBFST3 was between 20-60 pKat.mg$^{-1}$. The F3-ST exnibited two pH optima, 6.5 in bis-Tris and 8.5 in phosphate buffers. The cloned F3-ST showed substrate preference for quercetin and its 7-methyl derivative, rhamnetin (Table 1). The reaction product, when quercetin was used as substrate, co-chromatographed with authentic quercetin 3-sulfate (Fig. 1B, lane b). Control bacterial cultures, transformed with pBluescript SK$^-$, had no detectable ST activity. The Km values of the cloned F3-ST for the flavonol substrate quercetin and the sulfate donor PAPS were 0.3 and 0.4 $\mu$M, respectively. These Km values are in the same order of magnitude as those previously reported for the F. chloraefolia F3-ST (Varin and Ibrahim, 1992).

**D.2 Sequence analysis of pBFST3**

The cDNA insert of pBFST3 is 1211 bp long, excluding the poly A tail. It has an open reading frame beginning at nucleotide 55 and terminating at nucleotide 992, which encodes a putative protein of 312 amino acids with a predicted molecular mass of 36 kD (Fig. 2).

The F. bidentis F3-ST (pBFST3) nucleotide sequence has 92.5% identity with the F. chloraefolia F3-ST clone (pFST3), and 73.4% identity with the F. chloraefolia F4'-ST clone (pFST4') (Varin et al., 1992). The deduced amino acid sequence of pBFST3 shows 95.5% and 71.2% identity with those of pFST3 and pFST4', respectively (Fig. 3).

**D.3 F3-ST activity during growth of F. bidentis cell cultures**

Growth of F. bidentis cell culture was monitored by determining the fresh weight and protein content over a period of 14 d (Fig. 4). Cultures grown in nipple flasks showed a sigmoidal growth curve and cells reached the stationary phase after 10 d. The activity of F3-ST activity reached a maximum 2 d after subculture, and gradually decreased up to d 14.

**D.4 Effects of 2,4-D on F3-ST activity**

Preliminary experiments to study the effect of 2,4-D on F3-ST activity were carried out using batch cultures in Erlenmeyer flasks. The activity of F3-ST in the cell cultures elevated with an increasing concentration of 2,4-D, reaching a maximum at 25 $\mu$M, and declined at higher auxin levels (Fig. 5A). Cell cultures grown in nipple flasks and treated with 25 $\mu$M 2,4-D also showed a rapid increase of F3-ST activity within the first 3 h and reached a maximum at 24 h, as compared with the control cultures (Fig. 5B).

To determine whether the increase of F3-ST activity in

response to 2,4-D was a consequence of an increased level of gene expression, two approaches were used. The first approach was to examine the level of F3-ST mRNA transcripts in response to exposure to 2,4-D. Northern blot analysis was performed using pBFST3 as a probe. A transcript of 1,300 bp was detected by this probe; its level increased within 3 h of exposure of the cultured cells to 2,4-D and reached a maximum of ca. 10-fold increase 24 h after treatment, then showed a subsequent slow decrease (Fig. 5C).

In another experiment, batch cultures (grown in 250-mL Erlenmyer flasks) were pre-treated with the RNA polymerase inhibitor, actinomycin D (0.1 mM) 3 h prior to the addition of 2,4-D. F3-ST activity observed under these conditions was lower by ca. 50% 15 h, and by ca. 70% 24 h after the addition of auxin as compared with controls containing no actinomycin D (Fig. 6). The latter exhibited maximum enzyme activity 12 h after addition of 2,4-D, unlike 24 h maximum observed in cultures grown in 1-L nipple flasks (Fig. 5B).

**D.5 Effect of flavonols on F3-ST activity**

The effects of quercetin and its sulfated derivatives (quercetin 3-sulfate and quercetin 3,7,4'-trisulfate) on enzyme activity were determined during culture growth. Quercetin sulfate levels of 3 μM were high relative to the Ki for the enzyme (0.25 μM; Varin and Ibrahim, 1992), but within the range of concentrations of these compounds found in F.

bidentis. Values for endogenous sulfated flavonoids vary from 30 μM in the apical bud to 1 μM in the third leaf (Hannoufa et al., 1991). Whereas quercetin had no detectable effect on the activity profile of F3-ST, both flavonol sulfate esters reduced enzyme activity to approximately 50% of those levels observed with the control cultures (Fig. 7A). The decline in F3-ST activity in response to either quercetin 3-sulfate or quercetin 3,4,7'-trisulfate does not seem to be a consequence of a change in the F3-ST gene expression, because the levels of the enzyme-specific transcript were relatively stable during the treatment period (Fig. 7B). However, more frequent sampling might reveal fluctuation, especially prior to d 3.

**E. Discussion**

In this study, we describe the isolation, characterization and expression of a cDNA clone encoding the F3-ST from F. bidentis (pBFST3). Furthermore, the effects of 2,4-D as well as both the enzyme substrate, quercetin, and the sulfated products, quercetin 3-sulfate and quercetin 3,7,4'-trisulfate, on the activity of the F3-ST in F. bidentis were investigated.

Sequence analysis of the pBFST3 clone revealed high sequence similarity with the F3-ST and moderate levels of similarity to the F4'-ST genes from F. chloraefolia at both the nucleotide and amino acid levels (Varin et al., 1992), although the enzyme exhibited lowest affinity to kaempferol.

The Km values for both quercetin and PAPS, as well as the pH optima of F. bidentis F3-ST expressed in E. coli were quite similar to those reported for the F. chloraefolia F3-ST (Varin and Ibrahim, 1992). However, the substrate preference of the cloned F. bidentis F3-ST differs from that of F. chloraefolia F3-ST in that the former enzyme accepted quercetin, rhamnetin and isorhamnetin but not kaempferol, as substrates. The latter substrates were previously reported to be sulfated by cell-free extracts of both F. bidentis and F. chloraefolia (Varin et al., 1987). In addition, the relative sulfate acceptor ability of rhamnetin and isorhamnetin in that study also differed from that reported here. However, the two studies are not strictly comparable, because the former study measured ST activity in cell-free plant extracts, whereas the present report used a cloned F3-ST that was expressed in E. coli and assayed using the desalted bacterial cell lysate. It may be that either the E. coli expression system is affecting the substrate specificity of the cloned enzyme, or that the kaempferol-sulfating activity observed in cell-free extracts is due to another enzyme.

Study of the regulation of F3-ST expression in F. bidentis cell cultures showed that the F3-ST activity increased 2 to 3 d after subculture, although a corresponding accumulation of flavonol sulfate esters was not detected. The transient increase in enzyme activity is probably due to the transfer of cultured cells to fresh media (Fig. 4) by the so

called "dilution effect" which is characterized by a short lag period (Kuhn et al., 1984). These results are similar to those reported for the early enzymes of the phenylpropanoid pathway, especially PAL, in both parsley and soybean cell cultures (Hahlbrock et al., 1980).

In this study, F. bidentis cell cultures grown in nipple flasks and treated with 2,4-D showed an increase in F3-ST activity. Northern blot analysis revealed that the increase in F3-ST activity was concomitant with an increase in F3-ST transcript levels. Both the enzyme activity and mRNA transcript levels increased within 3 h of treatment with 2,4-D. F3-ST activity reached a maximum at 24 h and declined gradually up to 48 h, though they remained several fold higher than control levels throughout this time course. Transcript levels reached a maximum at 24 h after treatment and then declined slightly (Fig. 5C). When cell cultures were grown in Erlenmyer flasks, F3-ST activity reached a maximum somewhat earlier in response to 2,4-D treatment (Fig. 6).

It is possible that part of the signal on the northern blots (Fig. 5D, 7B) could be due to the cross-hybridization of the pBFST3 probe with mRNAs of other, as yet uncloned, STs. However, this source of error seems unlikely; intact tissues of F. bidentis contain position-specific ST activities other than the F3-ST (Varin and Ibrahim, 1991), no such activity was detected in F. bidentis cell cultures including those treated with 2,4-D. The comparison of the DNA sequence between STs

with different position-specific activities isolated from the same species show similarities. The pFST3 and pFST4' from F. chloraefolia have 76% overall sequence similarity, and two clones from F. bidentis, pBFST3 and pBFSTX (a FST-like cDNA clone with, as yet, uncharacterized enzymatic activity) have 74% overall sequence similarity. The 200 bp internal region with the highest sequence similarity between pBFST3 and pBFSTX are 78.5% identical. If similar levels of sequence identity exist between STs with different position-specific activities, cross hybridization of the pBFST3 probe to other flavonol STs would not be expected under the stringency conditions that were used. Hybridization of pBFSTX to northern blots of total RNA of 2,4-D treated cell cultures gave signals less than 10% of the signal from pBFST3 (data not shown).

Pre-incubation of the cultured cells with the transcriptional inhibitor, actinomycin-D reduced the 2,4-D-dependent induction of F3-ST activity (Fig. 6), as well as the level of immunoreactive protein (data not shown). These results suggest that enzyme induction resulting from exposure to 2,4-D is regulated at the level of gene expression. Northern blot analysis revealed that the level of F3-ST transcripts was increased ca. 10-fold following exposure to 25 $\mu$M 2,4-D, which is in contrast to the reported effect of 2,4-D on the early enzymes of the flavonoid pathway. In fact, 2,4-D has previously been reported to down-regulate PAL, CHS and chalcone-flavanone isomerase in carrot tissue cultures (Ozeki

et al., 1989, 1990). However, the differences in the reported effects of 2,4-D on gene expression in tissue cultures may be due to species specificity.

Recently, Mavandad et al. (1990) and Loake et al. (1991) have reported that the phenylpropanoid pathway intermediates, t-cinnamic acid and p-coumaric acid, regulate expression at the level of transcription for PAL, the first enzyme of the phenylpropanoid pathway and for CHS, the first committed enzyme in flavonoid biosynthesis. These results suggested that a regulatory mechanism may respond to a high concentration of pathway intermediates and thus, may control the rate of synthesis of the enzymes involved. In this study, the exposure of F. bidentis cultures to either quercetin 3-sulfate or quercetin 3,7,4'-trisulfate decreased F3-ST enzyme activity by approximately 50%, although the decrease might be partly due to inhibition by the product-uptake or the contamination of quercetin 3-sulfate in the protein preparation. The treatment with either of the sulfated compounds had no effect on F3-ST transcript levels. Moreover, quercetin 3-sulfate has been demonstrated to inhibit F3-ST activity in a non-competitive manner in vitro (Varin and Ibrahim, 1992), suggesting that the regulation of this enzyme by the sulfated product is not at the transcriptional level, but is likely at the enzymatic level.

It has been reported that flavonol aglycones may act as inhibitors of polar auxin transport and that flavonol

conjugates could act as antagonists of this inhibition. Some flavonoid aglycones (quercetin, kaempferol or apigenin) bind to the NPA receptor, inhibit the efflux of auxin from the basal end of stem cells, thereby inhibiting polar transport and causing intracellular accumulation (Jacobs and Rubery, 1988). In contrast, flavonol conjugates, including quercetin 3-sulfate, have been shown to strongly bind the NPA receptor and block the quercetin-stimulated accumulation of auxin (Faulkner and Rubery, 1992). The fact that F3-ST gene expression is up-regulated by 2,4-D, suggests that the polar transport of auxin may be autoregulated at the level of gene expression. In such a model, high levels of auxin would cause increased levels of F3-ST activity and result in the depletion of quercetin and in increased levels of 3-sulfated flavonols. Another point of regulation of auxin transport is that flavonol sulfates inhibit F3-ST activity. Both of these changes would tend to function as a balancing component of the regulation of auxin transport. The high concentration of both sulfated flavonols and ST enzyme activity in shoot tips, and their lower concentration in older tissues in this species (Hannoufa et al., 1991) are consistent with the role of a regulator that would stimulate auxin transport from the apical tissues. Although flavonoid aglycones and their conjugated forms are of widespread occurrence in plants, some of them, including sulfated flavonoids, have limited taxonomic distribution. However, in species where they do occur, such

as Flaveria spp., sulfated flavonols may be involved in an interactive mechanism for both the positive and negative control of auxin transport. The intracellular localization of both conjugated and unconjugated flavonols at the ultrastructural level is necessary to evaluate this model. This remains an important area for further investigation.

## F. Literature Cited


Aviv H, Leder P (1972) Purification of biologically active globin mRNA by chromatography on oligothymidylic acid cellulose. Proc Natl Acad Sci USA 69: 1408-1412

Barron D, Varin L, Ibrahim RK (1986) Identification and biosynthesis of glucosylated and sulfated flavonols in Flaveria bidentis. Z. Naturforsch. 41c: 813-819

Barron D, Varin L, Ibrahim RK, Harborne JB, William CA (1988) Sulfated flavonoids - an update. Phytochemistry 27: 2375-2395

Bleichert E, Varin L, Hannoufa A, Ibrahim RK (1989) In vitro culture of Flaveria spp. and its potential for the biosynthesis of sulfated flavonoids. Plant Physiol 89: S-187

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72: 248-254

Faulkner IJ, Rubery PH (1992) Flavonoids and flavonoid sulfates as probes of auxin-transport regulation in Curcubita pepo hypocotyl segments and vesicles. Planta 186: 618-625

Gulick PJ, Dvorak R (1990) Selective enrichment of cDNAs from salt-stress-induced genes in the wheatgrass Lophopyrum elongatum by the formamide-phenol emulsion reassociation technique. Gene 95: 173-177

Hahlbrock K, Schroder J, Vieregge J (1980) Enzyme regulation in parsley and soybean cell cultures. In A Fiechter, ed, Advances in Biochemical Engineering, Vol 18. Springer-Verlag, London, pp 39-60

Hannoufa A, Varin L, Ibrahim R (1991) Spatial distribution of flavonoid conjugates and their enzymes in Flaveria bidentis. Plant Physiol 97: 259-263

Harborne JB (1975) Flavonoid sulphates: A new class of natural product of ecological significance in plants. In L Reinhold, JB Harborne, eds, Progress in Phytochemistry, Vol 4. Pergamon Press, New York, pp 189-208

Ibrahim RK, Varin L (1993) Flavonoid enzymology. In P.J. Lea, ed, Methods in Plant Biochemistry, Vol 9. Academic Press, New York, pp 99-130

Jacobs M, Rubery PH (1988) Naturally occurring auxin transport regulators. Science 241: 346-349

Kuhn D, Chappell J, Boudet A, Hahlbrock K (1984) Induction of phenylalanine ammonia-lyase and 4-coumarate:Co A ligase mRNAs in cultured plant cells by UV light or fungal elicitor. Proc Natl Acad Sci USA **81**: 1102-1106

Louke GJ, Choudhary AD, Harrison MJ, Mavandad M, Lamb CJ, Dixon RA (1991) Phenylpropanoid pathway intermediates regulate transient expression of a chalcone synthase gene promoter. Plant Cell **3**: 829-840

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissue. Anal Biochem **163**: 16-20

Mavandad M, Edwards R, Liang X, Lamb CJ, Dixon RA (1990) Effects of trans-cinnamic acid on expression of the bean phenylalanine ammonia lyase gene family. Plant Physiol **94**: 671-680

Ozeki Y, Matsuoka M, Ohashi Y, Kano-Murakami Y, Yamamoto N, Tanaka Y (1989) Induction and suppression of anthocyanin synthesis in carrot suspension cell cultures regulated by 2,4-D, In WGW Kurz, ed, Primary and Secondary Metabolism of Plant Cell Cultures. Springer-Verlag, Berlin, pp 102-109

Ozeki Y, Komamine A, Tanaka Y (1990) Induction and repression of phenylalanine ammonia-lyase and chalcone synthase enzyme proteins and mRNAs in carrot cell suspension cultures regulated by 2,4-D. Physiol Plant **78**: 400-408

Sambrook J, Fritsch, EF, Maniatis T (1989) Molecular cloning, a laboratory manual. Second edition, Cold Spring Harbor Laboratory Press, New York

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain termination inhibitors. Proc Natl Acad Sci USA **74**: 8073-8077

Varin L (1992) Flavonoid sulfation: phytochemistry, enzymology and molecular biology. Rec Adv Phytochem **26**: 233-252

Varin L, Ibrahim RK (1989) Partial purification and characterization of three flavonol-specific sulfotransferases from Flaveria chloraefolia. Plant Physiol **90**: 977-981

Varin L, Ibrahim RK (1991) Partial purification and some properties of flavonol 7-sulfotransferase from Flaveria bidentis. Plant Physiol **95**:1254-1258

Varin L, Ibrahim RK (1992) Novel flavonol 3-sulfotransferase: purification, kinetic properties, and amino acid sequence. J Biol Chem **267**: 1858-1863

Varin L, Barron D, Ibrahim RK (1987a) Enzymatic synthesis of sulphated flavonols in Flaveria. Phytochemistry **26**: 135-138

Varin L, Barron D, Ibrahim RK (1987b) Enzymatic assay for flavonoid sulfotransferase. Anal Biochem **161**: 176-180

Varin L, DeLuca V, Ibrahim R, Brisson N (1992) Molecular characterization of two plant flavonol sulfotransferases. Proc Natl Acad Sci USA **89**: 1286-1290

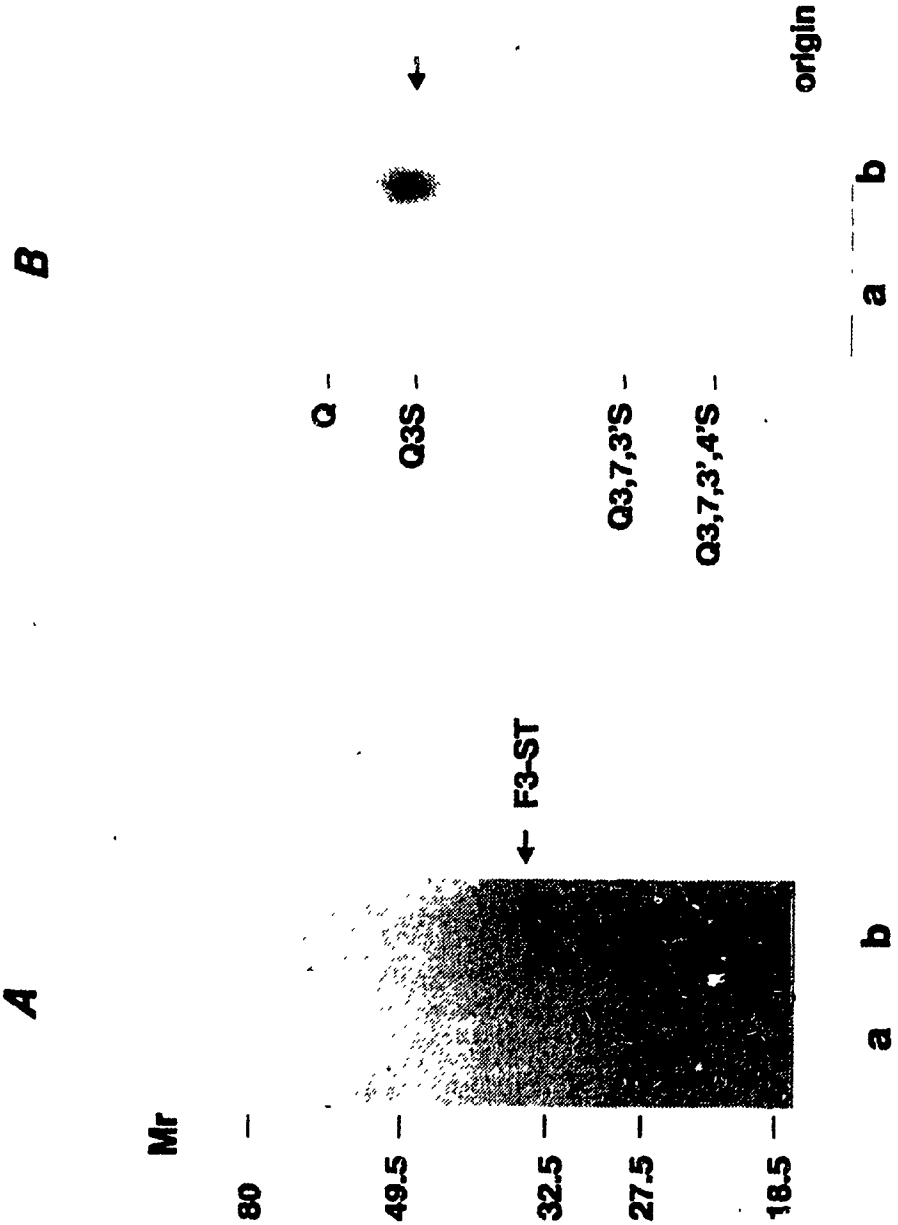
**Table 1.** Substrate specificity of F3-ST expressed in *E. coli*.

| Substrate | Relative activity[1,2] |
|---|---|
| Quercetin | 100 |
| Rhamnetin | 75 |
| Isorhamnetin | 10 |

[1] F3-ST activity with quercetin as substrate is 50 pKat.mg$^{-1}$

[2] Naringenin, luteolin, kaempferol, tamarixetin, quercetin 3-sulfate, kaempferol 3-sulfate and tamarixetin 3-sulfate did not act as sulfate acceptors.

**Figure 1.** (A) Western blot analysis of lysates from IPTG induced E. coli cultures: (a) control containing pBluescript $SK^-$ and (b) pBFST3. Blot was probed with a polyclonal antibody raised against F. chloraefolia F3-ST. The arrow indicates an immunoreactive band, corresponding to F3-ST. (B) Autoradiogram of the chromatographed enzyme reaction products from the lysate of E. coli control cultures after incubation with quercetin as substrate and PAPS as co-substrate (lane a), and that expressing the cDNA clone pBFST3 (lane b). The relative migration of reference compounds are marked at the left: Q, quercetin; Q3S, quercetin 3-sulfate; Q3,7,3'S, quercetin 3,7,3'-trisulfate and Q3,7,3'4'S, quercetin 3,7,3',4'-tetrasulfate. Labeled spot in lane b comigrates with quercetin 3-sulfate.


A
Mr
80
49.5
32.5
27.5
18.5
F3-ST
a
b
B
Q
Q3S
Q3,7,3'S
Q3,7,3',4'S
origin
a
b

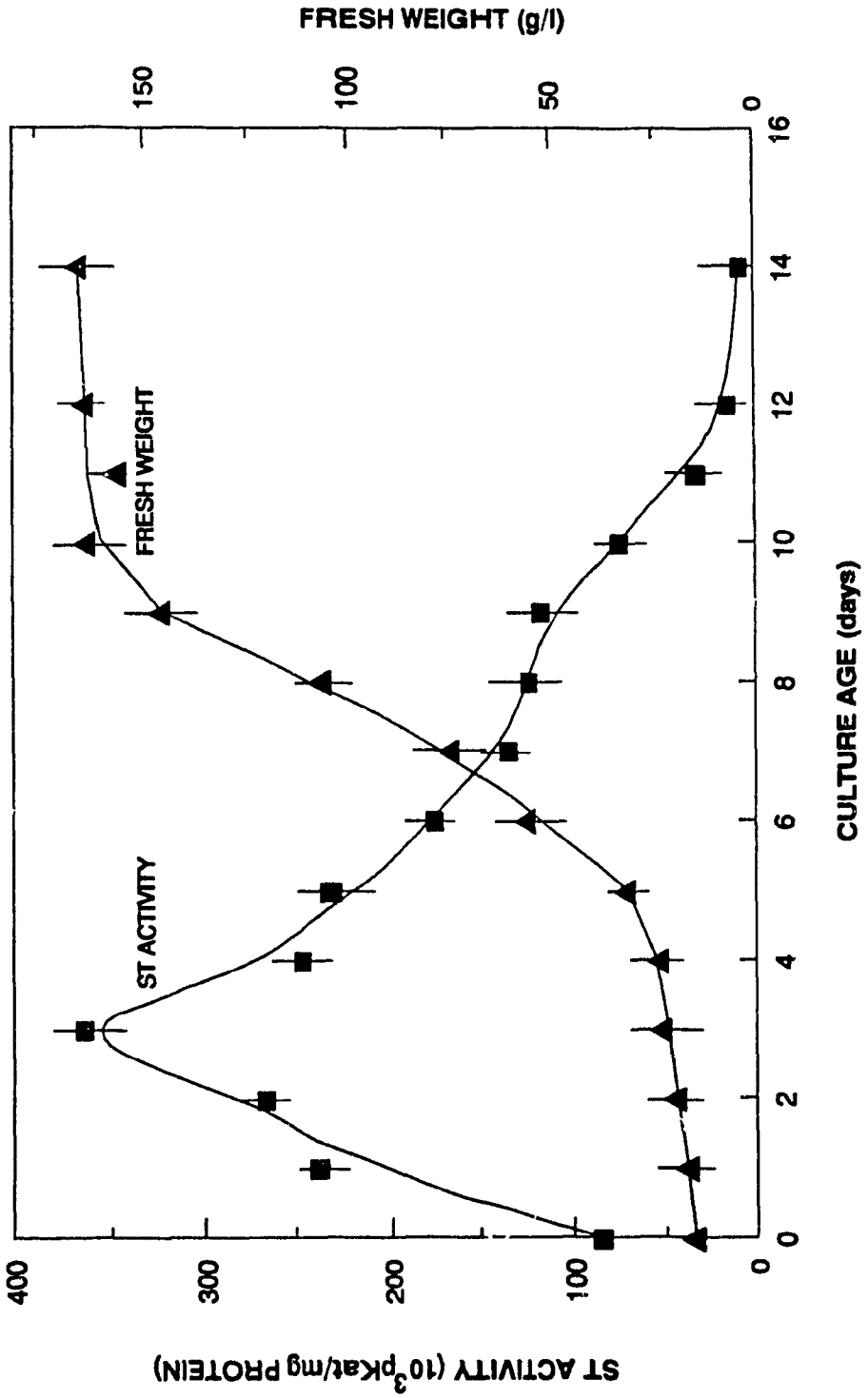
```
     TCTCGATCTATCACAAAAGTATTGAACTTCCTATTTGTATCATTTCGATCGATTATGGAG
1                                                          M  E
     GATATTATCAAAACACTTCCACAACACACTTGTAGCTTTTTGAAACAAAGGTTCACCTTG
3     D  I  I  K  T  L  P  Q  H  T  C  S  F  L  K  Q  R  F  T  L
     TACAAGTATCAAGACGTGTGGAATCATCAAGAGTTTCTTGAAGGACGAATGTTATCTGAA
23    Y  K  Y  Q  D  V  W  N  H  Q  E  F  L  E  G  R  M  L  S  E
     CAAACCTTCAAGGCACATCCCAATGATGTATTTCTCGCTAGTTATCCCAAAAGTGGCACG
43    Q  T  F  K  A  H  P  N  D  V  F  L  A  S  Y  P  K  S  G  T
     ACATGGCTAAAAGCCTTGGCATTTGCCATCATAACGCGAGAAAAGTTTGATGATTCCACA
63    T  W  L  K  A  L  A  F  A  I  I  T  R  E  K  F  D  D  S  T
     AGTCCTTTGCTCACAACCATGCCTCATGATTGCATTCCTCTCCTAGAGAAAGACCTTGAA
83    S  P  L  L  T  T  M  P  H  D  C  I  P  L  L  E  K  D  L  E
     AAAATTCAAGAAAACCAAAGGAACTCACTCTACACACCCATCTCGACACACTTTCATTAC
103   K  I  Q  E  N  Q  R  N  S  L  Y  T  P  I  S  T  H  F  H  Y
     AAATCCCTACCTGAGTCAGCCCGGACATCAAACTGCAAGATAGTTTACATATACCGGAAT
123   K  S  L  P  E  S  A  R  T  S  N  C  K  I  V  Y  I  Y  R  N
     ATGAAAGATGTCATTGTTTCTTATTACCATTTCCTGAGACAGATAGTTAAACTATCTGTG
143   M  K  D  V  I  V  S  Y  Y  H  F  L  R  Q  I  V  K  L  S  V
     GAAGAGGCCCCATTTGAGGAGGCAGTTGATGAGTTTTGTCAAGGTATTTCAAGTTGTGGA
163   E  E  A  P  F  E  E  A  V  D  E  F  C  Q  G  I  S  S  C  G
     CCATATTGGGAACACATCTTGGGATACTGGAAAGCAAGCTTGGAGAAGCCAGAGATATTT
183   P  Y  W  E  H  I  L  G  Y  W  K  A  S  L  E  K  P  E  I  F
     CTTTTCTTGAAATACGAAGACATGAAAAAAGATCCGGTACCAAGTGTGAAGAAACTTGCA
203   L  F  L  K  Y  E  D  M  K  K  D  P  V  P  S  V  K  K  L  A
     GATTTCATTGGGCATCCCTTTACACCCAAAGAAGAGGAAGCGGGTGTGATTGAAAATATT
223   D  F  I  G  H  P  F  T  P  K  E  E  E  A  G  V  I  E  N  I
     ATAAAGTTATGTAGTTATGAGAAATTAAGCAGCTTAGAAGTAAACAAAAGTGGAATGCAT
243   I  K  L  C  S  F  E  K  L  S  S  L  E  V  N  K  S  G  M  H
     CGTCCTGAAGAAGCTCATTCCATTGAAAACCGACTTTACTTTAGAAAGGGTAAAGATGGA
263   R  P  E  E  A  H  S  I  E  N  R  L  Y  F  R  K  G  K  D  G
     GATTGGAAGAACTACTTTACTGATGAGATGATTGAGAAAATAGACAAATTGATCGATGAA
283   D  W  K  N  Y  F  T  D  E  M  I  E  K  I  D  K  L  I  D  E
     AAATTAGGTGCCACTGGTTTAGTTCTAAAATAAAAGGCTCAAGATCATACTTTATTTGAG
303   K  L  G  A  T  G  L  V  L  K  -
     AGCAAAACAACTCGATTTCAAACTATCTTTCATTATTCTTGTTTTAAATAATTGAGGGCT
     AGGTCTCTTTTCAATTCTTTTCTTCATTATGTAATTTAATTTGGCCTTATGTTATTTTGT
     TGTATGTCTTATTAATAATGATTATTTTCTTTCTTGGAAGATTTGTTAAACCAAATTGAT
     AACTTTTGACCAAAAAAAAAAAAAAAAAAA
```

**Figure 2.** The nucleotide and amino acid sequences of pBFST3.

```
pBFST3                  MEDIIKTLPQHTCSFLKQRFTLYKYQDVWNHQEFLEGRML
pFST3                   ----------------H-------K-A----------I-
pFST4'          METTKTQFES-AEM--K-------S--G-I-------F-GL-NNI--AI-

pBFST3          SEQTFKAHPNDVFLASYPKSGTTWLKALAFAIITREKFDDSTSPLLTTMP
pFST3           ---K----------------------W♦IC-------------------
pFST4'          AQ-S---R-D----C---------------Y--V------EF------NI-

pBFST3          HDCIPLLEKDLEKIQENQRNSLYTPISTHFHYKSLPESARTSNCKIVYIY
pFST3           ----------------------------------------------------
pFST4'          -N---YI----K--V---N--CF--MA--MP-HV--K-ILAL---M----

pBFST3          RNMKDVIVSYYHFLRQIVKLSVEEAPFEEAVDEFCQGISSCGPYWEHILG
pFST3           ------------------------------F-----------------K-
pFST4'          --I------F---G-E-T--PL-D-------F---YH---QF----D-L--

pBFST3          YWKASLEKPEIFLFLKYEDMKKDPVPSVKKLADFIGhPFTPKEEEAGVIE
pFST3           ----------------------------------------------------
pFST4'          -------R--VI-------V----TSN--R--E---Y---FE--KE----

pBFST3          NIIKLCSFEKLSSLEVNKSGMHRPEEAHSIENRLYFRKGKDGDWKNYFTD
pFST3           D-V-------------------------------------------------
pFST4'          S--------N--N-------NSK♦♦GFLP---------A-----------

pBFST3          EMIEKIDKLIDEKLGATGLVLK    312
pFST3           --TQ----------------      311
pFST4'          --T----------S-------     320
```

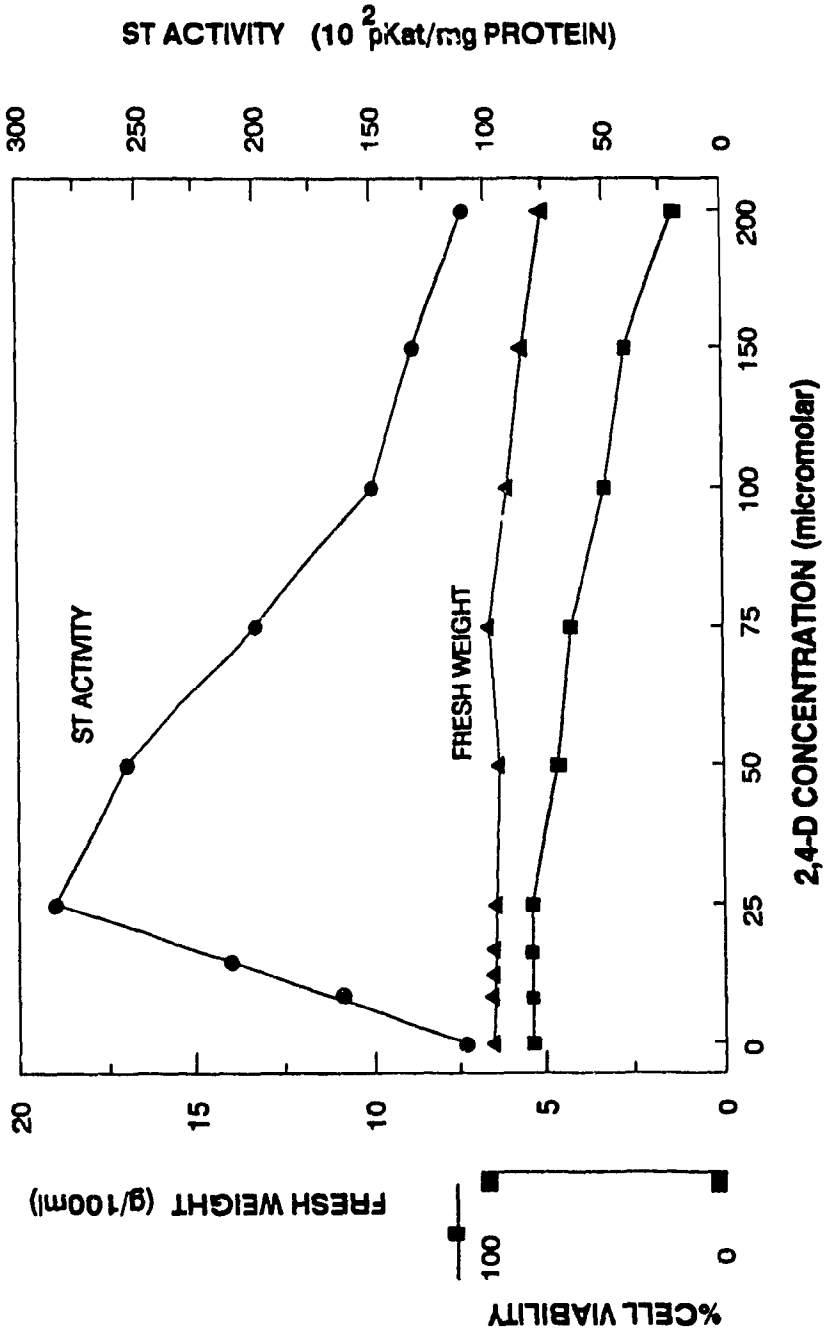
**Figure 3.** The amino acid sequence alignment of F. bidentis F3-ST (pBFST3), F. chloraefolia F3-ST (pFST3) and F. chloraefolia F4'-ST (pFST4'). The pBFST3 amino acid sequence shows 95.5% and 71.2% identity to pFST3 and pFST4', respectively. Bars (-) indicate identical amino acids, and diamonds (♦) indicate gaps in the aligned sequence.

**Figure 4.** Flavonol 3-sulfotransferase activity in relation to age of *F. bidentis* cell cultures.

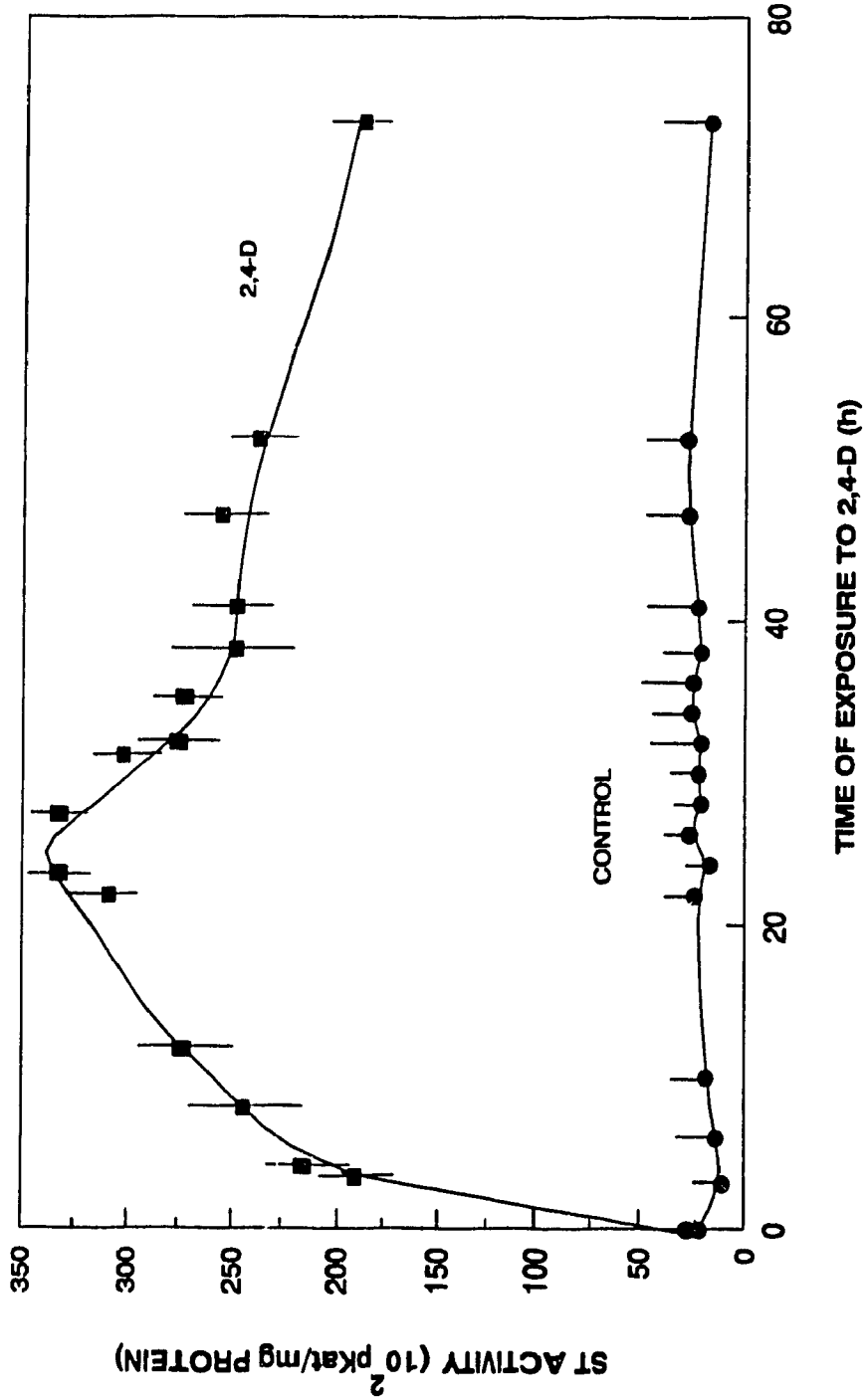

FRESH WEIGHT (g/l)
150
100
50
0
16
14
12
10
8
6
4
2
0
CULTURE AGE (days)
FRESH WEIGHT
ST ACTIVITY
400
300
200
100
0
ST ACTIVITY ($10^3$pKat/mg PROTEIN)

4

**Figure 5.** (A) Dose-response curve. Ten-day-old cultures, grown in 250-mL Erlenmeyer flasks, were treated with the indicated concentrations of 2,4-D, harvested 24 h after treatment, and cell-free extracts were assayed for F3-ST activity. (B) Time-response curve. Ten-day-old cultures, grown in 1-L nipple flasks, were treated with 25 $\mu$M 2,4-D; F3-ST activity was monitored for 72 h. (C) Northern blot analysis of BF3ST gene product from cell cultures. Ten-day-old cultures, grown in 1-L nipple flasks, were treated with 2,4-D treatment or were untreated (2,4-D substituted by water). Lanes contain 5 $\mu$g of total RNA taken at the indicated time points. Blots were probed with cDNA clone, pBFST3, labeled with $^{32}P$ by the random primer method (Amersham).


ST ACTIVITY (10 $^{2}$pKat/mg PROTEIN)
300
250
200
150
100
50
0
ST ACTIVITY
FRESH WEIGHT
20
15
10
5
0
FRESH WEIGHT (g/100ml)
0
25
50
75
100
150
200
2,4-D CONCENTRATION (micromolar)
100
0
%CELL VIABILITY

54


ST ACTIVITY ($10^2$ pkat/mg PROTEIN)
350
300
250
200
150
100
50
0
0
20
40
60
80
TIME OF EXPOSURE TO 2,4-D (h)
2,4-D
CONTROL

*5B*

*5C*

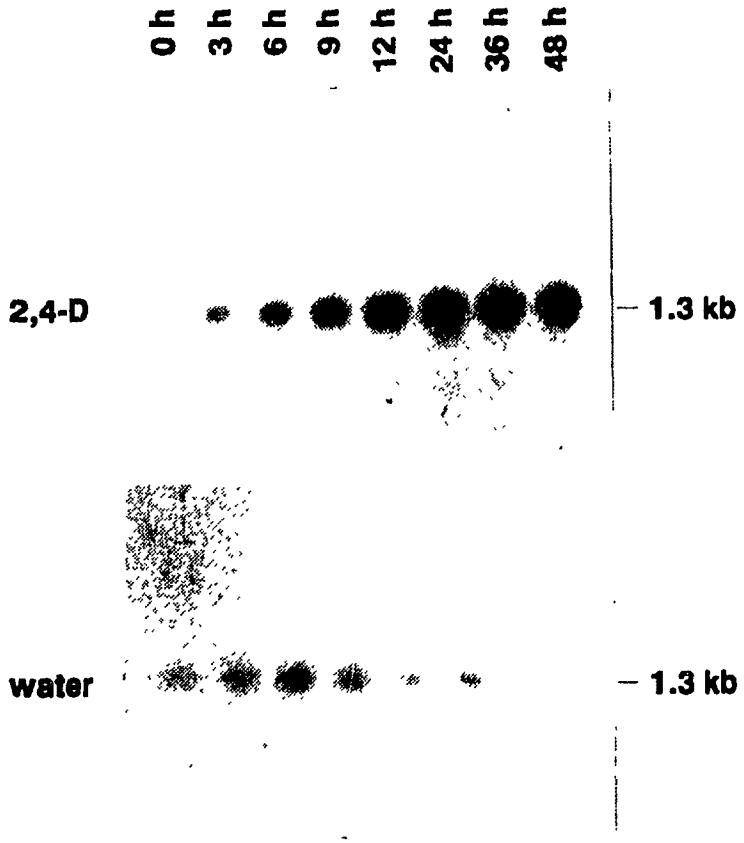
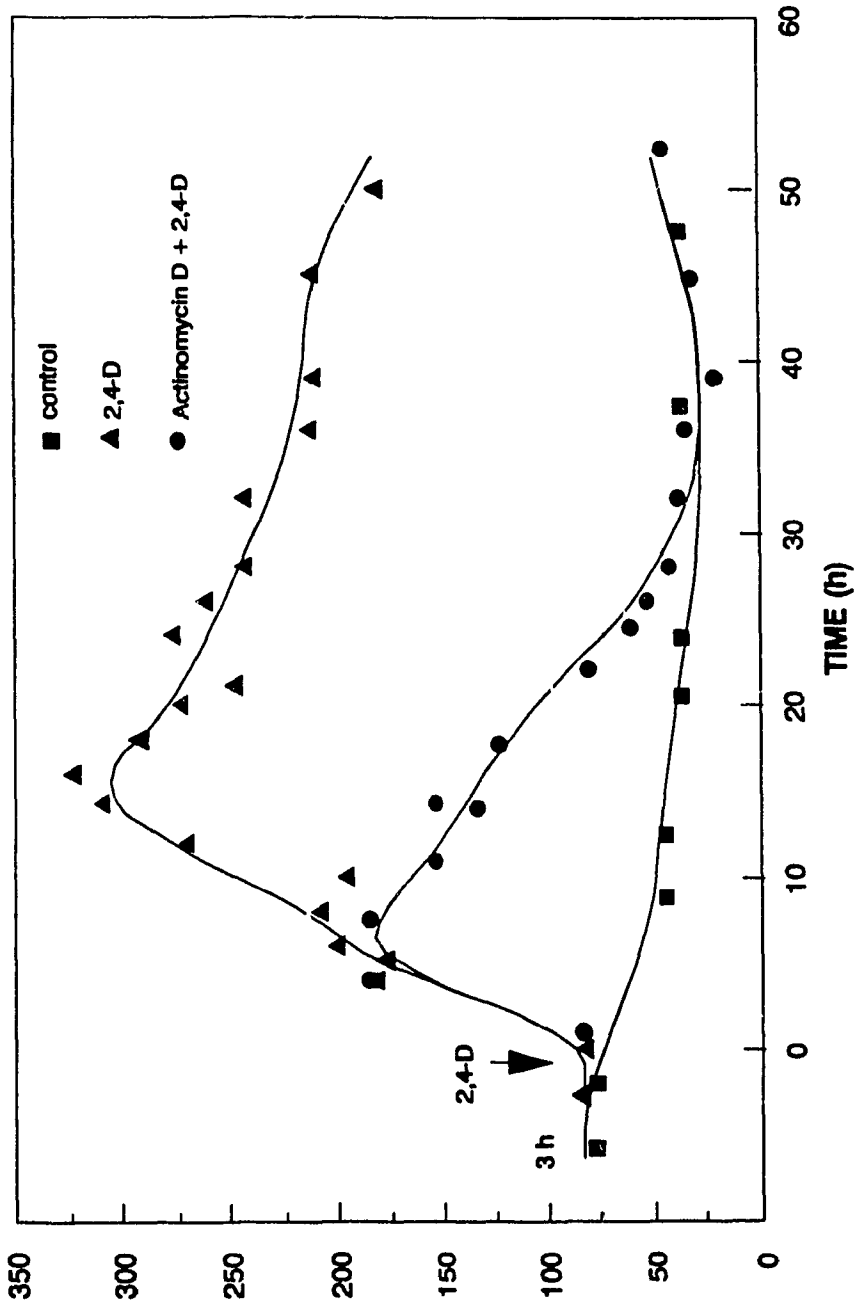
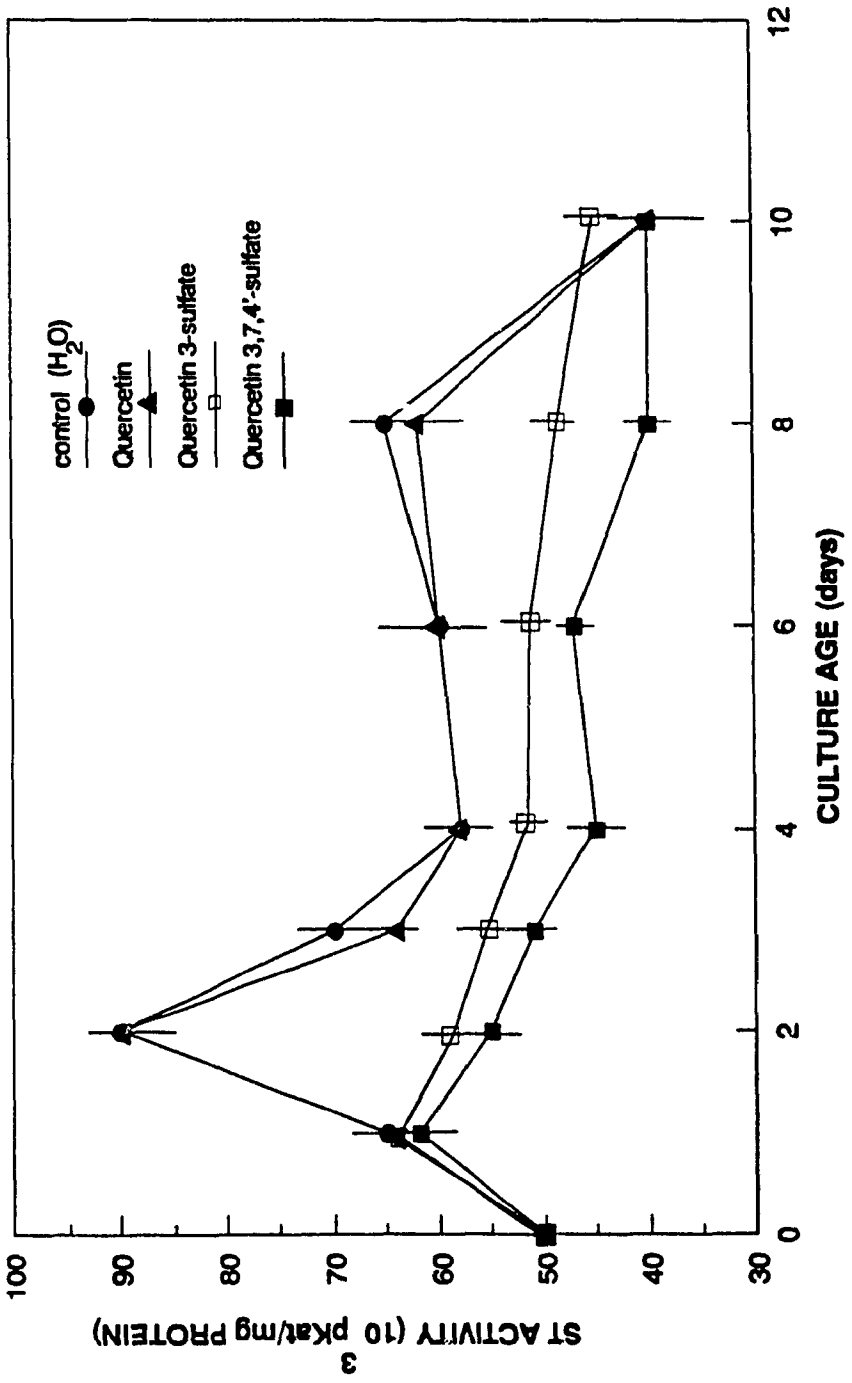


**Figure 6.** F3-ST induction kinetics in response to 2,4-D and actinomycin D treatments. Cell cultures grown in 250-mL Erlenmeyer flasks were either treated with actinomycin D for 3 h prior, or not, and subsequently treated with 25 $\mu$M 2,4-D.


control
2,4-D
Actinomycin D + 2,4-D
2,4-D
3 h
TIME (h)
ST ACTIVITY (10² pKat/mg PROTEIN)
0
50
100
150
200
250
300
350
0
10
20
30
40
50
60

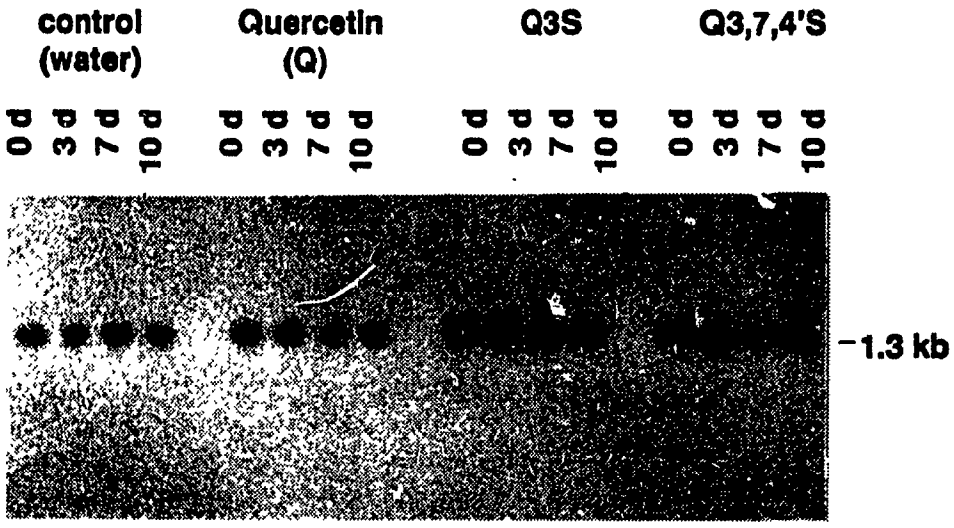
9

**Figure 7.** The effect of different flavonols on F3-ST activity. (A) Ten-day-old cultures from 250 mL Erlenmyer flasks were transferred to media containing 3 μM of quercetin (-▲-), quercetin 3-sulfate (- -) or quercetin 3,7,4'-trisulfate (- -) as compared to control (-●-). These were subsequently sampled and assayed for F3-ST activity. Bars indicate standard deviations calculated from triplicate experiments. (B) Northern blot analysis of cell cultures following treatment with quercetin (Q), quercetin 3-sulfate (Q3S) and quercetin 3,7,4'-trisulfate (Q3,7,4'S). A control treatment substituted water for the flavonols. Samples were collected on days 0, 3, 7, and 10. Each lane was loaded with ten μg of total RNA. Blots were probed with pBFST3. Each treatment should be compared to its own d 0, control, as the darkening from left to right on the blot is likely an artifact. The blot was exposed to Kodak XAR film five times longer than the northern blots shown in Fig. 5C.


control ($H_2O$)
Quercetin
Quercetin 3-sulfate
Quercetin 3,7,4'-sulfate
ST ACTIVITY ($10^3$ pkat/mg PROTEIN)
CULTURE AGE (days)
100
90
80
70
60
50
40
30
0
2
4
6
8
10
12

*7A*

*7B*


control
(water)
Quercetin
(Q)
Q3S
Q3,7,4'S
0d
3d
7d
10d
0d
3d
7d
10d
0d
3d
7d
10d
0d
3d
7d
10d
1.3 kb

## CHAPTER IV

# Molecular Cloning and Characterization of Flavonol Sulfotransferase-Like cDNA from *Flaveria bidentis*

## A. Abstract

A flavonol sulfotransferase-like cDNA clone (pBFSTX) was obtained from *Flaveria bidentis* by a polymerase chain reaction (PCR)-mediated strategy. Synthetic oligonucleotides, based on the conserved amino acid sequences among reported sulfotransferases, were used to amplify a gene fragment from the genomic DNA of *F. bidentis* which corresponds to the internal portion of a sulfotransferase-like protein. Gene specific oligonucleotides were designed and used to obtain the 3' and 5' cDNA ends by PCR amplification. The full length cDNA was reconstructed and expressed in *E. coli*. This clone is 1,141 bp with a single open reading frame of 927 bp. The deduced amino acid sequence (309 aa) of this cDNA clone exhibits between 80 and 82% similarity to the reported flavonol sulfotransferases (F-STs) of *Flaveria spp*. The clone was expressed in *E. coli* and yeast, and the gene product was detected by western blot analysis. BFSTX transcripts of 1.2 kb were detected by northern blot hybridization. The members of the flavonol sulfotransferase gene family in *F. bidentis* were estimated by Southern blot analysis using pBFSTX and the previously reported flavonol 3-sulfotransferase cDNA (pBFST3).

**B. Introduction**

Sulfotransferases (STs) catalyse the transfer of the sulfate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to the hydroxyl group of various substrates. The enzymes represent a heterogenous family of enzymes that show different substrate specificities. In mammalian systems, enzymatic sulfation has been implicated in the regulation of transport and the metabolism of steroid hormones, heparan sulfate biosynthesis, and aryl- and phenol detoxification reactions (Borthwick et al., 1993; Orellana and Hirschberg, 1994; Weinshilboum and Otterness, 1994; Wood et al., 1994).

Shoot tissues of Flaveria spp. (Asteraceae) accumulate a variety of flavonol sulfates whose biosynthesis is catalysed by a number of position-specific F-STs (Varin, 1992). The distribution of F-STs and the differential accumulation of sulfated flavonols in young shoot, but not in root tissues of F. bidentis, (Hannoufa et al., 1991) indicate that the encoding STs have developmental and tissue-specific regulation. We have recently shown that the expression of flavonol 3-sulfotransferase (F3-ST) is regulated by auxin and that it is possibly involved in the regulation of polar auxin transport (Chapter III in this dissertation and Ananvoranich et al., 1994).

To date, two cDNA clones encoding F3-ST from F. chloraefolia (Varin et al., 1992) and F. bidentis (Ananvoranich et al., 1994), and another encoding F4'-ST from

F. chloraefolia (Varin et al., 1992) have been isolated and characterized. Work presented in this chapter concerns the characterization of a flavonol sulfotransferase-like cDNA clone (pBFSTX) from F. bidentis.

## C. Materials and Methods

### C.1 Plant materials

Flaveria bidentis var. angustifolia O.K. and its cell suspension cultures were propagated as previously described (Ananvoranich et al., 1994).

### C.2 Chemicals

3'-Phosphoadenosine 5'-phospho-[35S]sulfate (PAPS, 1.57 Ci/mmol) was purchased from New England Nuclear (Boston, MA), and TBADP was from Aldrich Chemical Co. (Milwaukee, WI). The immunodetection kit was obtained from Bio-Rad (Mississauga, ONT) and UniZap XR cDNA library synthesis kit was from Stratagene (La Jolla, CA). Sequenase Version 1.0 sequencing kit was from USB (Cleveland, OH). Restriction endonucleases and all other modification enzymes were from BioCan (Mississauga, ONT). Oligonucleotide primers were ordered from Dalton Chemical (York, ONT).

### C.3 cDNA library construction and pBluescript excision

Poly(A)$^+$ RNA was isolated from terminal buds of F.

bidentis, and a cDNA library was constructed in lambda UniZap XR (Ananvoranich et al., 1994). Massive in vivo excision of a plasmid version of the library in the pBluescript SK⁻ vector was obtained from the lambda Zap XR library with the ExAssist/SOLR system according to the manufacturer's instructions (Stratagene).

**C.4 Plant genomic DNA isolation**

Frozen plant tissue was ground to a fine powder and gently mixed with a buffer containing 10 mM Tris-HCl (pH 9.4), 10 mM EDTA, 80 mM KCl, 4 mM spermidine, 4 mM spermine, and 0.5 M sucrose. The mixture was filtered through 4 layers of cheesecloth, and Triton X-100 was added to the filtrate to a final concentration of 0.5% v/v. After incubation on ice for 15 min, the nuclei were pelleted by centrifugation at 1670 g for 20 min, then resuspended and lysed by incubation at 65°C for 30 min in a buffer containing 50 mM Tris-HCl, 100 mM NaCl, 100 mM EDTA, 0.1 mg/ml proteinase K, and 0.5% w/v SDS, pH 8.0. The lysate was extracted with one volume of phenol-chloroform-*iso*amylalcohol (25:24:1). The DNA-containing aqueous layer was recovered and precipitated by adding 0.1 vol of 3 M sodium acetate (pH 5.2) and 2 vol of ethanol and incubating on ice for 2 h. The precipitated DNA was pelleted by centrifugation at 12,000g and washed with 70% ethanol. The genomic DNA was dissolved in sterile distilled water, and treated with RNase for 2 h.

**C.5 Cloning and expression strategy**

A PCR-mediated cloning strategy was employed for the isolation of flavonol sulfotransferase cDNA clones from F. bidentis. Four conserved regions of ST's were identified by comparing deduced amino acid sequences of plant sulfotransferases: F3-ST, F4'-ST of F. chloraefolia (Varin et al., 1992), F3-ST of F. bidentis (Ananvoranich et al., 1994), and mammalian phenol-, aryl-, estrogen- and hydroxysteroid sulfotransferases (Fig. 1). Two synthetic oligonucleotides, SP-1 and SP-2 (Table 1) corresponding to regions I and IV were made and used to amplify a fragment from the genomic DNA of F. bidentis which was then cloned into pBluescript SK⁻ vector.

The nucleotide sequence of this fragment (pBFSTX-gen) was determined and then used to design gene specific primers: ST-A, ST-B, ST-C, and ST-D (Table 1). ST-A primer was used with pBluescript vector specific primer, f-vec (Fig. 2) and Pfu DNA polymerase (Stratagene) to amplify and clone the 3'-end of the gene from the plasmid version of the cDNA library. Similarly, ST-C primer was used with pBluescript vector specific primer, r-vec (Fig. 2) to amplify and clone the 5'-end of the gene from the cDNA library. The PCR amplified portions of the cDNA clones were ligated into pBluescript SK⁻ vector and further selected by colony hybridization. Those clones whose sequences overlapped that of the partial length genomic clone (pBFSTX-gen) were sequenced entirely in both directions. pST-3' containing 766 bp of the 3'-end portion and pST-5'

containing 934 bp of the 5'end portion of the pBFSTX (Fig. 2) were selected for a reconstruction of a full length cDNA (pBFSTX). The 5'-end portion of BFSTX was cleaved from pST-5' using SacI and StyI. The 5'-end fragment was then purified using a Qiagen DNA fragment isolation kit and ligated into pST-3' plasmid-digested with SacI and StyI. Sequence at the junction of the reconstructed pBFSTX was verified by sequencing using synthetic primers ST-A and ST-B.

The ST-E primer, corresponding to nucleotides 28-54 of the pBFSTX was used with f-vec primer to introduce a BamHI site just upstream of the ATG start codon of BFSTX. This subclone was amplified directly from the cDNA library using Pfu DNA polymerase (Table 1, Fig. 2). The PCR fragment was isolated after agarose gel electrophoresis, purified by a QiaGen DNA fragment isolation kit and cloned into pBluescript $SK^-$.

The full coding region and 3'-UTR, referred to as BFSTX-s, was also subcloned into E. coli expression vectors (pGEX-3x, and pTrc-His) and yeast expression vectors pYes-I and pYes-II (generous gifts from Dr. Adrian Tsang and Dr. Luc Varin, respectively). Yeast expression vector construction were performed as described in the manufacturer's instruction (InVitrogen).

**C.6 DNA Sequencing**

DNA sequence was obtained from a number of independently

derived PCR fragments of the gene. These include (a) sequences from the ends of the clones from pBFSTX-gen, the PCR fragment derived from genomic DNA. The nucleotide sequence corresponds to nucleotide 217 to 895 of the full length cDNA; (b) end sequences and internal sequences from clone pST-3', the PCR fragment derived from the cDNA library using ST-A and f-vec primers. The sequence was obtained with vector primers (reverse and universal primers) and synthetic primers ST-A, ST-B, and ST-C. (c) end sequences and internal sequences from clone pST-5', the PCR fragment derived from the cDNA library using ST-C and r-vec primers. The sequence was obtained with vector primers and synthetic primers ST-A, ST-B, ST-C, ST-E, and ST-D. (d) end sequences and internal sequences from four independent isolates of pBFSTX-s, the PCR fragment derived from the cDNA library using ST-E and f-vec primers. These nucleotide sequences were obtained with pBluescript vector primers and synthetic primers ST-A, ST-B, ST-C, ST-D. (e) end sequences and internal sequences from subclones obtained following EcoRI digestion of pBFSTX. The pBFSTX plasmid was digested with EcoRI. Following an EcoRI inactivation by heating at 65°C for 30 min, the reaction mixture was diluted 10 times, and a 1 $\mu$L aliquot was taken for a ligation reaction using T4 DNA ligase. Subclones were obtained for sequencing reaction using vector primers and synthetic primers ST-B and ST-C. The relative position of sequencing primers as well as the internal EcoRI site are shown in Fig. 2. Sequence from

various regions of the clones were obtained from at least two independent clones. Subclones of the coding sequence and 3'-UTR region (pBFSTX-s) in pBluescript $SK^-$ and pTrc-His were sequenced through ligated junctions using vector specific primers. Sequence analysis was performed using the GCG software, version 7 (Genetics Computer Group, 1991).

**C.7 Polymerase chain reaction (PCR)**

A 100 $\mu$L reaction contained 10o ng of genomic DNA or 10 ng of cDNA of F. bidentis cDNA plasmid library DNA as template, 20 pmol of each primer, 50 $\mu$M each dNTP, and Pfu DNA polymerase (Stratagene). The primers used for amplification are listed in Table 1. The reaction buffer contains 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5-2.0 mM $MgCl_2$, and 0.1% Triton X-100, and overlaid with 75 $\mu$l mineral oil. The PCR reaction was carried out on a Thermocycle apparatus (Interscience, ONT) with the following temperature cycle: denaturing 95°C (1 min), annealing 52-55°C (1 min), and extension 72°C (0.5 min). These cycles were repeated for 30 times with a 5-min extension at 72°C following the final cycle. The reaction products were analyzed by agarose gel electrophoresis and by Southern blots.

**C.8 Cloning PCR fragments**

Following gel electrophoresis, selected PCR fragments were isolated and purified by QiaGen DNA fragment isolation

kit (Qiagen). The amount of DNA recovered was quantified either by UV spectrophotometric absorption or by visualization after ethidium bromide staining on thin agarose gels. An optimum amount of PCR fragment was ligated to pBluescript SK$^-$ vector by T4 DNA ligase (BioCan), and transformed into E. coli strain XL1-Blue by electroporation or by heat shock.

**C.9 Colony hybridization**

Colonies of transformed E. coli were screened by colony hybridization. pBFST3 was used as a probe for a selection of cloned PCR fragments generated from genomic DNA; the insert of pBFSTX-gen was used as a probe for a selection of cloned PCR amplified fragments from a cDNA library. Transformants were grown overnight at 37°C on an LB plate containing an appropriate antibiotic. The lift was performed by placing a nylon membrane on the plate for one min. The membrane was then placed on 3 MM Whatman paper saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 7 min and subsequently transferred to a neutralizing solution (0.5 M Tris-HCl, pH 7.2, 1.5 M NaCl) two times for 3 min each. The DNAs were fixed on the membrane by baking at 80°C for 2 h. The membrane was incubated with 100 µg/ml Proteinase K in Tris-borate buffer (0.09 M Tris-borate, 0.002 M EDTA) at 37°C for 1 h. Following Proteinase K treatment, the membranes were air-dried, rinsed with 95% ethanol, then with chloroform, then air-dried prior to Southern blot hybridization.

**C.10 Southern blot analysis**

Genomic DNA isolated from terminal buds of F. bidentis was digested with the restriction endonucleases HaeIII, HindIII, and EcoRI. Ten μg aliquots of digested DNA were fractionated by gel electrophoresis through 1% agarose gel using a non-denaturing running buffer, Tris-borate pH 8.5. The fractionated DNAs were visualized under UV illumination following ethidium bromide staining. The gel was then placed in the denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 min at room temperature, then transferred to a neutralizing solution (0.5 M Tris-HCl, pH 7.2, 1.5 M NaCl, 1 mM EDTA) twice for 15 min. The DNA was transferred to Hybond-N nylon membrane (Amersham) by capillary blotting (Sambrook et al., 1989), and fixed to the membrane by baking at 80°C for 2 h. Prehybridization and hybridization for Southern blots were performed as described by Gulick and Dvorak (1990). The blot was probed with [$^{32}$P]-labelled DNA probe, prepared from XbaI-XhoI fragment of pBFSTX. To detect additional genes of F-STs, a similar blot was prepared and probed with [$^{32}$P]-labelled DNA probe, prepared from the XbaI-XhoI fragment of pBFST3, a cDNA clone of the F3-ST from F. bidentis. Blots were then washed 3 times with a solution containing 0.1x SSC, 1% w/v SDS and 0.1% w/v sodium pyrophosphate, for 20 min at 55°C. The blots were exposed to Fuji RX film with an intensifying screen at -80°C for various periods of time. The sizes of DNA fragments

were estimated by comparison with molecular weight markers, either 1 kb ladder (Gibco BRL) or HindIII-EcoRI digested lambda DNA markers (BioCan).

**C.11 Northern blot analysis**

Northern blot analysis was carried out to determine the size of transcript encoding BFSTX and the level of BFSTX gene expression, as compared with those of BFST3. Poly(A)$^+$ RNA was prepared from F. bidentis terminal buds and two-day-old cell culture (Ananvoranich et al., 1994). The two tissues were chosen because they exhibit the highest F-ST enzyme activity (Hannoufa et al., 1991; Ananvoranich et al., 1994). RNA concentration was quantified by UV absorbance at 260 nm (Sambrook et al. 1989). Equal amounts (1 $\mu$g) of poly(A)$^+$ RNA were fractionated on 1% agarose formaldehyde gels using an electrophoresis buffer containing 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0. After formaldehyde gel electrophoresis, the fractionated RNA was transferred onto Hybond-N nylon membrane (Amersham), and fixed on the nylon membrane by UV crosslinking. Prehybridization and hybridization for northern blots were performed as described by Gulick and Dvorak (1990). [$^{32}$P]-Labelled DNA probes were prepared from XbaI-XhoI fragments of pBFSTX and pBFST3. Following hybridization, the membranes were washed 3 times with a solution containing 0.1x SSC, 1% w/v SDS and 0.1% w/v sodium pyrophosphate, for 20 min at 55°C. The blots were

exposed to Fuji RX film at -80°C for an appropriate time. The sizes of the RNAs were estimated by comparison with RNA molecular weight markers (Gibco BRL).

**C.12 Preparation of protein extracts from E. coli and yeast**

One hundred fifty μL of bacterial overnight cultures, grown in LB medium with tetracycline (100 $\mu g.mL^{-1}$) and ampicillin (50 $\mu g.mL^{-1}$), were used to inoculate 3 mL of the same medium. IPTG was added after 1 h to a final concentration of 1 mM. The induced cells were harvested by centrifugation (10,000xg), and the cell pellets were resuspended in 1 mL of an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 14 mM ß-ME, then lysed by sonication. Protein preparations were used for F-ST assay and western blot analysis.

For pGEX-3x expression system, a fusion gene product of glutathione S-transferase was subjected to a cleavage using factor Xa (New England Biolab). The factor Xa cleaves the fusion product right at the junction of the two moieties. For pTrc-His expression system, the gene product was added 36 amino acids, including a run of 6 consecutive histidine to the N-terminal portion. Gene products which were obtained from cultures carrying pTrc-His construct were purified by nickel-sepharose column (InVitrogen), and enterokinase digestion (Boehringer-Mannheim). The purification would remove the 36 amino acid fusion from the N-terminal portion of the protein.

Ten mL of yeast cultures, grown in YEPD medium (InVitrogen instruction manual), was used to inoculate 100 mL of YEP medium containing 3% w/v galactose. After 16 h incubation, the cultures were centrifuged (5,000g), and cell pellets were resuspended in 2 mL of an extraction buffer containing 100 mM phosphate buffer pH 7.0, 0.1 mM PMSF, 1 mM EDTA, and 1 mM EGTA, then lysed by vortexing with glass beads. The lysates were used for F-ST assay and western blot analysis. Protein concentrations were determined according to the method of Bradford (1976) using the BioRad reagent, and BSA as the protein standard.

**C.13 Sulfotransferase assay**

The method described by Varin et al. (1987) was used with some modification to optimize the assay conditions. The reaction mixture contained 1 $\mu$M flavonol substrate, 0.1 $\mu$Ci [$^{35}$S]PAPS (1 $\mu$M) and up to 60 $\mu$g protein. The flavonol substrates: quercetin, quercetin 3-sulfate, quercetin 3,3'-disulfate, kaempferol, kaempferol 3-sulfate, tamarixetin, tamarixetin 3-sulfate, rhamnetin and isorhamnetin were used for the characterization of cDNA clones. The reaction mixture was adjusted to 100 $\mu$L with the assay buffers. Different assay buffer systems, 100 mM bis-Tris (pH 6.0, 6.5, and 7.0), 100 mM Tris-HCl (pH 7.0, 7.5, 8.0) and 20 mM phosphate buffer (pH 5.0, 6.0, 7.0, 8.0), were used with or without 1 mM of divalent ions ($Mg^{2+}$, $Ca^{2+}$, or $Mn^{2+}$) and 0.1 mM of protease

inhibitors (PMSF, EDTA, and EGTA). The reaction mixture was incubated for 15 min at 30°C, and stopped by the addition of 20 µl of 20% v/v acetic acid, 20 µL of 0.1 M TBADP and 250 µL ethyl acetate. The sulfated reaction products were extracted in the ethyl acetate layer, of which an aliquot was counted for radioactivity in a toluene-based scintillation fluid. The remaining sample was used for the identification of enzyme reaction products by co-chromatography with reference compounds and autoradiography on X-ray film.

### C.14 Western blot analysis

Protein extracts (40 µg) were fractionated by SDS-PAGE using 12% acrylamide gels. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes using a semi-dry electrotransfer apparatus according to the manufacturer's instructions (BioRad). The blots were developed with anti-flavonol 3-ST immune serum (1:2000) or nonimmune serum (1:2000) as primary antibody, then with alkaline phosphatase-conjugated anti-rabbit IgG as the secondary antibody.

## D. Results

### D.1 Molecular cloning and characterization of a flavonol sulfotransferase-like cDNA clone (pBFSTX)

Clone BFSTX-gen obtained from F. bidentis genomic DNA by

PCR had high sequence similarity to F-ST's previously cloned from Flaveria spp. The 684 bp fragment clone contained a single continuous ORF and no intronic sequence. Gene specific primers, ST-A and ST-C were derived from the BFSTX-gen sequence and were used to obtain two overlapping cDNA clones, pST-3' and pST-5', from a cDNA library. The clones, which encompass the full coding region of the cDNA as well as the 3'- and 5'-UTR, were used to reconstructed a full length cDNA clone, pBFSTX. The insert of pBFSTX is 1,141 bp in length. It contains an ORF beginning at nucleotide 43 and terminating at nucleotide 970 which has 74.5% nucleotide sequence similarity to the F. bidentis F3-ST (pBFST3), 70.3% similarity to the F. chloraefolia 3-ST clone (pFST3), and 72.8% similarity to the F. chloraefolia 4'-ST clone (pFST4') (Varin et al., 1992). The deduced amino acid sequence of pBFSTX encodes a 309 amino acid protein which has 80.9%, 80.2% and 81.6% similarities with pBFST3, pFST3, and pFST4', respectively (Fig. 4). The pBFSTX encoded protein has a predicted molecular mass of 35917 Da, and an isoelectric point of 5.66.

Although the full length clone of pBFSTX has 5'-UTR containing TAA stop codon in-frame with the ATG start codon of lacZ in the pBluescript SK$^-$ (Stratagene) vector, it was expressed as described in the Methods section. Lysates obtained from IPTG-induced cultures were assayed for F-ST enzyme activity. A very low level of activity, about 4 times

the level of background (400 dpm/20 μg protein), was observed in assays which were carried out in Tris-HCl, pH 7.5 using kaempferol 3-sulfate, as substrate. In order to improve the level of expression, the 5'-UTR of pBFSTX was removed from the clone, and the first ATG of the cDNA was placed in-frame with the ATG start codon of pBluescript SK⁻ expression vector (pBFSTX-s). However, the activity of the clone could neither be reproduced, nor increased to a higher activity for further characterization. The insert of pBFSTX-s was also subcloned into both pGEX-3x (Pharmacia), pTrc-His (InVitrogen) and subsequently transformed into E. coli XL1-Blue strain. E. coli cultures containing pGEX-3x and pTrc-His with BFSTX were observed to produce a high level of gene products following the IPTG induction. The lysates prepared from IPTG-induced cultures, containing the BFSTX-s in pBluescript SK⁻, pGEX-3x and pTrc-His were subjected to western blot analysis. Blots were developed using a polyclonal antibody raised against F. chloraefolia F3-ST. The antibody reacted with a 36 kD protein band in the lysates of cells carrying pBluescript SK⁻ construction (Fig 5, lane d), a 55 kD protein band in the lysates of cells carrying pGEX-3x construction (Fig. 5, lane f), and a 36 kD protein band in the lysate of cells carrying pTrc-His construction (Fig. 5, lane h). Lysates of E. coli expressed cultures were then prepared from IPTG-induction for F-ST enzyme assay. No enzymatic activity could be detected using various assay conditions. Since the pGEX-3x expression

system resulted in a fusion gene product of BFSTX-s and glutathione S-transferase, lysates prepared from IPTG-induced cultures containing pGEX-3x with BFSTX-s were subjected to a protein cleavage by factor Xa (New England Biolab), which cleaves the fusion product at the junction of the two moieties. The F-ST activity in the cleavage products could not be detected. Lysates were prepared from cultures carrying pTrc-His with BFSTX-s were purified by nickel-sepharose column (InVitrogen), and enterokinase digestion (Boehringer-Mannheim), which removes the 36 amino acid fusion from the N-terminal portion of the protein. Protein fractions obtained following purification and digestion were assayed using various conditions and flavonol substrates; no activity was observed.

The BFSTX-s insert was also subcloned into yeast expression vectors, pYes-I and pYes-II. The gene products from the galactose-induced yeast cultures carrying these plasmid-bearing BFSTX-s exhibited no F-ST activity. Western blot analysis of the lysates of galactose-induced yeast cultures revealed low molecular weight bands (less than 25 kDa), after detection with the F3-ST antibody (data not shown).

**D.2 Northern and Southern blot analysis**

The BFSTX probe detected a transcript of 1.2 kb, in the poly(A)$^+$ RNA preparation from the terminal buds but no

transcript was detected in cell cultures (Fig. 6A). The BFST3 detected a transcript of 1.3 kb in poly $(A)^+$ RNA preparations from both terminal buds and from cell cultures.

Both BFSTX and BFST3 probes detected multiple bands with various intensities in HaeIII, HindIII, and EcoRI-digested genomic DNA. The internal sequence of BFSTX contains recognition sites for HindIII and EcoRI; that of BFST3 shows recognition sites for HaeIII and HindIII. At least three bands were detected in each digested DNA by both probes. The BFSTX probe detected multiple bands at estimated sizes ranging from 8.2, to 1.8 kb in HaeIII-digested DNA; from 9.6 to 0.46 kb in HindIII-digested DNA and from 11 to 3.2 kb in EcoRI-digested DNA (Fig. 7A). The BFST3 probe detected multiple bands at estimated sizes ranging from 5.3 to 0.5 kb in HaeIII-digested DNA; from 7.5 to 1.5 kb in HindIII-digested DNA and from 11 to 1.2 kb in EcoRI-digested DNA (Fig. 7B). Both probes revealed few bands with similar sizes; a 3.2-kb band in HaeIII-digested DNA, 11-kb, 6.8-kb and 3.2-kb in EcoRI-digested DNA. Therefore, at least three to five members of F-ST-like genes are apparently present in F. bidentis genome.

**E. Discussion**

Previous attempts to isolate the pBFSTX cDNA clone using a F3-ST polyclonal antibody screening of a F. bidentis cDNA expression library (Ananvoranich et al., 1994) were not successful, suggesting that BFSTX might be present in a very

low abundance in the cDNA library, and in the poly(A)$^+$ RNA isolated from the terminal bud tissue. The frequency of a lone of a particular gene in a cDNA library depends on the abundance of its mRNA in the tissue from which the library was constructed. However, since members of a gene family are expected to be present in equal frequency in a sample of genomic DNA, we initiated a search for additional genes of F-STs by PCR amplification of genomic DNA sequences. A fragment (BFSTX-gen) corresponding to one half of the internal region of the coding sequence of a ST-like gene was initially isolated from genomic DNA by PCR amplification. With subsequent PCR amplification from a cDNA library, cDNA corresponding to the full coding sequence of the gene were also isolated.

The amino acid sequences of reported ST cDNA clones allowed classification of STs into three families: phenol-, hydroxysteroid- and flavonol STs (Weinshilboum and Otterness, 1994). cDNA clones encoding STs from the same family share more than 60% identity. The amino acid sequence of pBFSTX shows less than 50% identity to aryl-, phenol STs, and steroid STs (Genetics Computer Group, 1991). Whereas the pBFSTX exhibits amino acid sequence identity of 61% and 67%, and similarity of 80.2% and 81.6% to the reported Flaveria F3-ST (pFST3), F4'-ST (pFST4'), respectively; the previously reported pBFST3 shows amino acid sequence identity of 96% and 71%, and similarity of 98% and 83% to pFST3 and pFST4',

respectively. Although the amino sequence of pBFSTX has less identity and similarity to reported Flaveria F-ST cDNA clones as compared to pBFST3, the high similarity (ca 80%) of pBFSTX to other reported F-STs strongly suggests that pBFSTX is a member of the F-ST family.

Subclones used for expression studies in pBluescript SK$^-$ were amplified directly from a cDNA library and ligated to expression vector. Three E. coli and two yeast expression vectors were used to assay expression and enzymatic activity. The expressed gene products of these clones were detected by western blot analysis using a polyclonal antibody raised against F. chloraefolia F3-ST (Fig. 5). However the enzymatic activity could be detected only at a very low level (400 dpm/20 μg protein), and only from expression with pBluescript SK$^-$ construct when kaempferol 3-sulfate was used as substrate. These results can be considered suggestive but are not convincing since the previous reported pBFST3 exhibited F3-ST activity (15,000 dpm/20 μg protein), about 200 times higher than background when quercetin was used as substrate. Various flavonol substrates and assay conditions were used for the pBFSTX enzyme characterization. The lack of detectable F-ST activity was surprising since other ST clones, pBFST3 of F. bidentis, pFST3 and pFST4' of F. chloraefolia, produced active gene products when they were expressed with pBluescript SK$^-$ in E. coli. In the yeast expression system, only small protein products could be detected, suggesting that degradation of the

gene product occurred either *in vivo* or *in vitro* prior to F-ST assay and western blot analysis. However, with the *E. coli* expression vectors, products of the expected molecular weight were detected. The fact that F-ST enzyme activity of pBFSTX could not be resolved raises a possibility that pBFSTX might catalyse the sulfation of substrates other than flavonols, however, sequence similarity with the *Flaveria* F-ST clones suggests otherwise. Other possibilities responsible for the failure to detect BFSTX enzyme activity in *E. coli* might also be the result of missing co-factors, problems of compartmentation, or protein processing steps that are present in the intact plant system but are different or lacking in *E. coli* system. Another approach to characterize the enzyme activity of this clone could be an introduction of BFSTX under either the control of a 35S-promoter, or an ubiquitin promoter into plant tissue by particle bombardment. Expression of the gene in plant tissue might allow the characterization of position-specific enzymatic activity of pBSFTX.

Because PCR amplification can introduce sequence changes into amplified DNA, it is also possible that mutations were introduced into the gene during the cloning procedures. However, a number of precaution were taken to insure fidelity in the reported sequences and in the clones used for expression studies. Amplification of the products used for sequence analysis, pBSFTX-gen, pST-3', and pST-5' were generated with a high fidelity heat stable DNA polymerase, Pfu

(Stratagene). Multiple independent clones were sequenced such that at least two independent clones contributed sequence to each region of the sequence (see Fig. 2).

Southern blot hybridization using BFSTX and BFST3 probes revealed multiple bands with various intensities in HaeIII, HindIII, and EcoRI-digested DNA (Fig. 7). The internal sequence of BFSTX contains recognition sites for HindIII and EcoRI; that of BFST3 shows recognition sites for HaeIII and HindIII. Although the pBFSTX-gen obtained from PCR fragment amplified of the genomic DNA contains no intronic sequence, no information concerning intronic sequence in BFST3 is available, as well as the information on other STs. A precise estimation of gene family member could not therefore be established. However, both probes detected bands with distinct sizes and some similar sizes; *e.g.* 3.2-kb band in HaeIII-digested DNA, 11-kb, 6.8-kb and 3.2-kb in EcoRI-digested DNA. Therefore, three to five numbers of F-ST-like genes are apparently present in F. bidentis genome. The presence of di-, tri-, and tetrasulfated flavonols in F. bidentis suggests that there are at least four F-STs corresponding to the specific substitution of 3-, 7-, 3'-, and 4'-positions of flavonols. Although different position specific STs have been identified from F. chloraefolia and F. bidentis (Varin et al., 1992; Ananvoranich et al., 1994), they account for the substitution of position 3, and 4' of flavonol to give rise to flavonol mono- and disulfates. It has yet not

been demonstrated that other sulfations are carried out by the position-specific STs. Characterization of the enzymatic activity of pBFSTX would, therefore, make an important contribution to the elucidation of substrate specificity of other STs.

## F. Literature Cited


Ananvoranich S, Varin L, Gulick P, Ibrahim R (1994) Cloning and regulation of flavonol 3-sulfotransferase in cell suspension cultures of Flaveria bidentis. Plant Physiol (in press)

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72: 248-254

Borthwick EB, Burchell A, Coughtrier MWH (1993) Purification and immunochemical characterization of a male-specific rat liver oestrogen sulfotransferase. Biochem J 289: 719-725

Genetics Computer Group (1991) Program Manual for the GCG package, Version 7, April 1991, Madison, WI

Gulick PJ, Dvorak R (1990) Selective enrichment of cDNAs from salt-stress- induced genes in the wheatgrass Lophopyrum elongatum by the formamide-phenol emulsion reassociation technique. Gene 95: 173-177

Hannoufa A, Varin L, Ibrahim R (1991) Spatial distribution of flavonoid conjugates and their enzymes in Flaveria bidentis. Plant Physiol 97: 259-263

Orellana A, Hirschberg C (1994) Molecular cloning and expression of a glycosamineglycan N-acetylglucosaminyl N-deacetylase/N-sulfotransferase from a heparin-producing cell line. J Biol Chem 269: 2270-2276

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual. Second edition, Cold Spring Harbor Laboratory Press, New York

Varin L (1992) Flavonoid sulfation: phytochemistry, enzymology and molecular biology. Rec Adv Phytochem 26: 233-252

Varin L, Barron D, Ibrahim RK (1987) Enzymatic assay for flavonoid sulfotransferase. Anal Biochem 161: 176-180

Varin L, DeLuca V, Ibrahim R, Brisson N (1992) Molecular characterization of two plant flavonol sulfotransferases. Proc Natl Acad Sci USA 89: 1286-1290

Weinshilboum R, Otterness D (1994) Sulfotransferase enzymes. In FC Kauffman, ed, Handbook of Experimental Pharmacology: Conjugation-Deconjugation Reactions in Drug metabolism and Toxicity,

Vol 112, Springer-Verlag, London, pp 45-78

Wood TC, Aksoy IA (1994) Human liver thermolabile phenol sulfotransferase: cDNA cloning, expression and characterization. Biochem Biophys Res Commun 198: 1119-1127

**Table 1.** Oligonucleotide primers used in cloning of pBFSTX

| Primer | Sequence (5'to 3') | Position[1] |
|---|---|---|
| SP-1[2] | TAA CCC AAA AGT GGC AC | 211-227 |
| SP-2[2] | GTA GTT CTT CCA ATC TCC | 877-895 |
| ST-A | CAA GCT TAC CGC TCG TGG | 375-392 |
| ST-B | GGT TTT GCC TGG CAT ATC | 632-649 |
| ST-C | GCC ACT TAG TTT CTC ATC | 935-952 |
| ST-D | CTT TCA CGG TTG TGT TC | 361-377 |
| ST-E[3] | TTT ATC AGG ATC CGT **ATG** GAA GAA ATC | 28-54 |
| r-vec[4] | AGC GGA TAA CAA TTT CAC ACA GG | - |
| f-vec[4] | TAG GGC GAA TTG GGT ACC G | - |

[1]Nucleotide position indicates the corresponding locations in pBFSTX.

[2]Oligonucleotide primers derived from conserved regions of reported sulfotransferases (Fig. 1, amino acid positions 67 to 72, and 296 to 301).

[3]A BamHI site (underlined) was introduced upstream of the start codon (ATG).

[4]Oligonucleotide primers corresponding to pBluescript nucleotide sequence flanking the multiple cloning to the 5'- and 3'-ends of the cDNA insert, respectively.

**Figure 1.** Amino acid alignment of reported sulfotransferases. The Flaveria flavonol sulfotransferases are pBFST3 (Genbank accession number **U10275**), pFST3 (**M84135**), pFST4'(**M84136**). Mammalian hydroxysteroid, and oestrogen sulfotransferases are pHeST (**L20000**), pHhST (**843859**), pMST (**L02335**), pRST (**D14987**), pBeST (**M54942**), and pReST (**M86758**). Mammalian phenol- and arylsulfotransferases are pHaST-a (**L19955**), pHpST (**L19999**), pHaST-b (**L19956**), and pRmRT (**L19998**). Numbers in brackets indicate deduced amino acid residues of ORFs. Conserved amino acids in sulfotransferases, from which the oligonucleotide primers for PCR were designed, are indicated in bold above the alignments (position 67 to 72, and 296 to 301). An identical amino acid is shown by a dash (-), and stop codons are shown by asterisk (*), and gaps introduced to obtain maximum similarity are indicated by diamonds (♦).

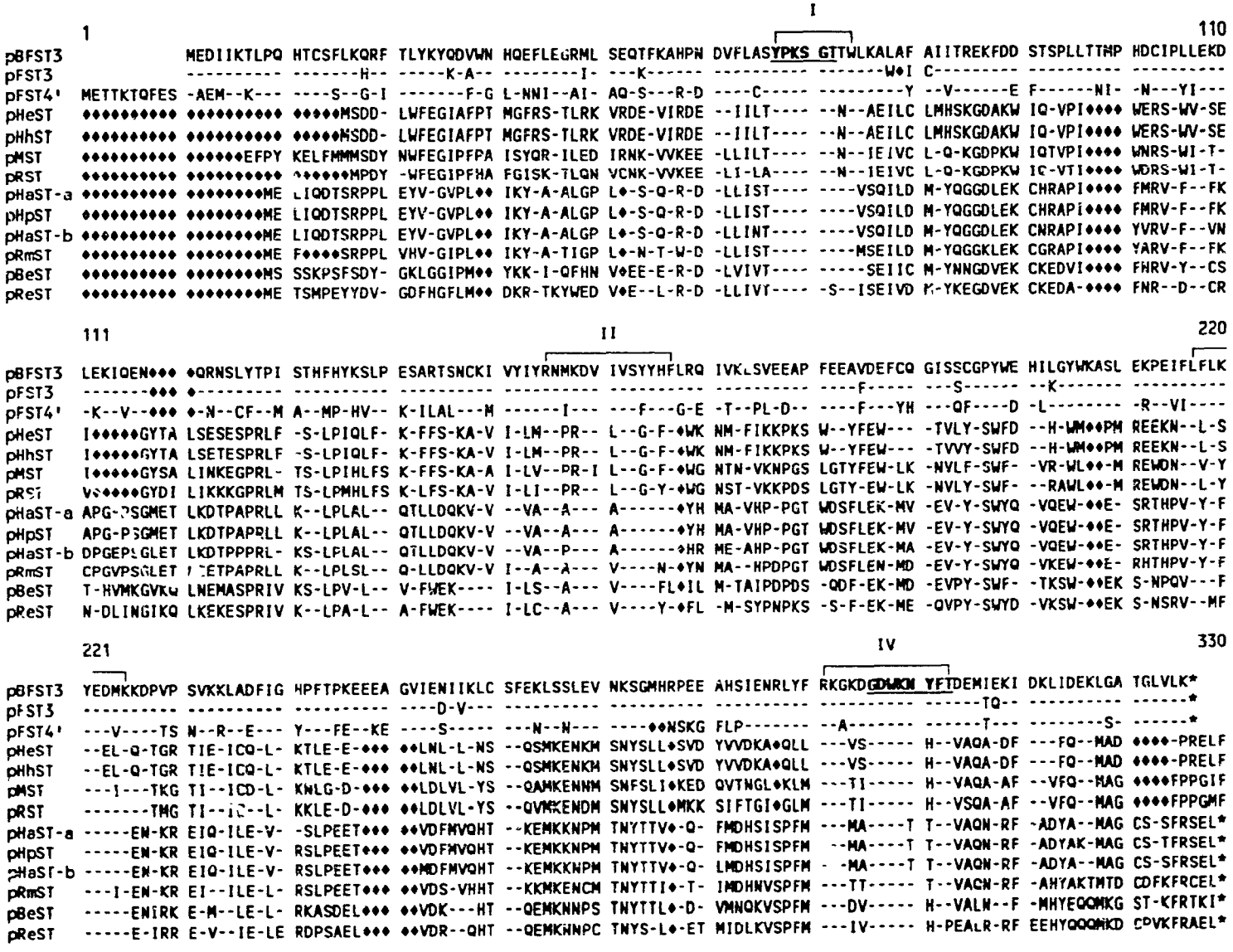
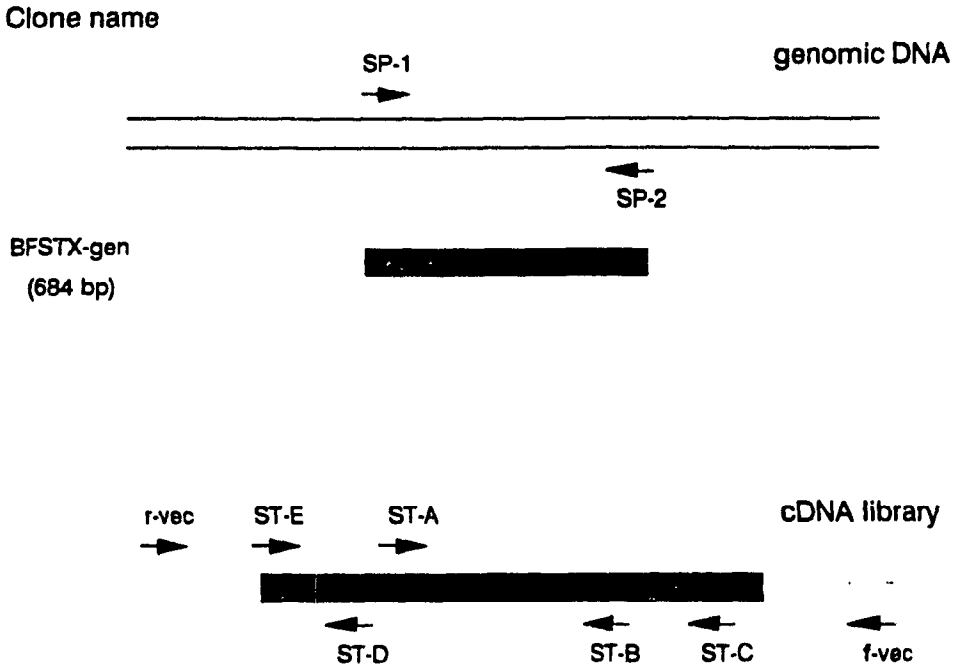
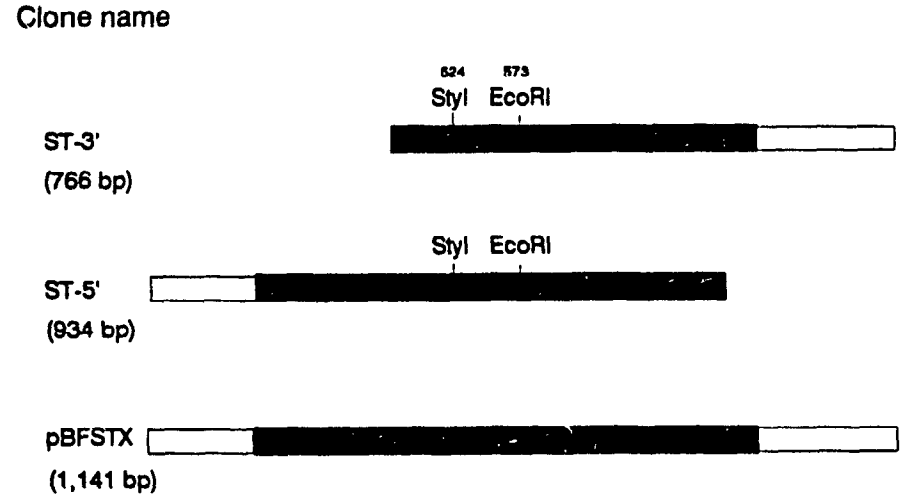


**Figure 2.** Schematic representation of the PCR-mediated cloning strategy for pBFSTX. Arrows indicate positions and orientation of oligonucleotide primers within the pBFSTX insert. The construction of a pBFSTX full length clone from clones pST-3' and pST-5' was carried out by using StyI (position 524) within the insert and multiple cloning sites of pBluescript SK$^-$.


Clone name
SP-1
genomic DNA
SP-2
BFSTX-gen
(684 bp)
r-vec
ST-E
ST-A
cDNA library
ST-D
ST-B
ST-C
f-vec


Clone name
524
573
StyI
EcoRI
ST-3'
(766 bp)
StyI
EcoRI
ST-5'
(934 bp)
pBFSTX
(1,141 bp)

```
     CCATAATCCACAACAAAAATTGTTAATTTTATCAGCTTCGGTATGGAAGAAATCTTAAAA
1                                                    M  E  E  I  L  K
     ACACTTCCACAACACACTTGTAGTTGGTTGAAACACAAGATAATCATGTACAAGTATCAA
7     T  L  P  Q  H  T  C  S  W  L  K  H  K  I  I  M  Y  K  Y  Q
     GACTTTTGGACATCTAAACAGTTGCTTGAGGGCACACTTATGGCTCAACAAAGCTTTAAG
27    D  F  W  T  S  K  Q  L  L  E  G  T  L  M  A  Q  Q  S  F  K
     GCTGAGCCTAGCGATGTATTTCTATGTAGCGCCCCCAAAACCGGCACAACTTGGCTAAAG
47    A  E  P  S  D  V  F  L  C  S  A  P  K  T  G  T  T  W  L  K
     GCATTGGCTTTTGCCATTGTAACCCGTGAAAATTTTGATGAATCCACAAGCCCATTGCTC
67    A  L  A  F  A  I  V  T  R  E  N  F  D  E  S  T  S  P  L  L
     AAAAAACTGGTTCATGAATGTGTTCCTTTCCTAGAGAGACAAGTTGAGGAAATTGAACAC
87    K  K  L  V  H  E  C  V  P  F  L  E  R  Q  V  E  E  I  E  H
     AACCGTGAAAGCTCAAGCTTACCGCTCGTGGCAACACACCTTCCTTATGCGTCATTGCCC
107   N  R  E  S  S  S  L  P  L  V  A  T  H  L  P  Y  A  S  L  P
     GAATCAGTCATAGCTTCAAACTGCAGGATGGTTTACATCTATCGGAACATAAAAGATGTC
127   E  S  V  I  A  S  N  C  R  M  V  Y  I  Y  R  N  I  K  D  V
     ATAGTTTCTAATTACCATTTCTTGAGAGAAGCGTTTAAACTATCCATGGAAGATGCACCG
147   I  V  S  N  Y  H  F  L  R  E  A  F  K  L  S  M  E  D  A  P
     TTTGAGGAGACATTTGAAGACTTCTATAACGGGAATTCAAGTTATGGTCCATACTGGGAT
167   F  E  E  T  F  E  D  F  Y  N  G  N  S  S  Y  G  P  Y  W  D
     CATATACTCGGATACCGGAAAGCAAGTCTTGATATGCCAGACAAAATACTTTTCTTGAAA
187   H  I  L  G  Y  R  K  A  S  L  D  M  P  D  K  I  L  F  L  K
     TATGAAGATTTGAAAAGTGAACCTATAAGCAATGTGAAGAGACTTGCAGAGTTCATCGGG
207   Y  E  D  L  K  S  E  P  I  S  N  V  K  R  L  A  E  F  I  G
     TATCCATTTTCAAACGATGAAGAGAAAGCAGGTGTGATTGAAAACATTATTAAAATGTGC
227   Y  P  F  S  N  D  E  E  K  A  G  V  I  E  N  I  I  N  M  C
     AGCTTTGAGAATCTGAGTAGCTTAGAAGTGAATAAAACTAGAAAGCCGAAGGGTGGTATG
247   S  F  E  N  L  S  S  L  E  V  N  K  T  R  K  P  K  G  G  M
     TTAGAAAATCGACTTTATTACAGGAAGGGACAAGACGGAGATTGGAAAAACTACTTTACT
267   L  E  N  R  L  Y  Y  R  K  G  Q  D  G  D  W  K  N  Y  F  T
     AATGAGATGAAAGAGAAGATCGATAAAATAATGGATGAGAAACTAAGTGGCACTGGTTTA
287   N  E  M  K  E  K  I  D  K  I  M  D  E  K  L  S  G  T  G  L
     ATTCTAAAATAAAAGCATCACACCATTATGTTGGAATTAGTGAATTTGTGTAGTTCTGAG
307   I  L  K  -
     TGTTTAATAAAAATGTGATTGTAATAGTGGCACAACTTGTTGTGTGTTTGTCTTCTATTT
     TGATGTAATTTTGATTGAATTTATAACTAGTTTGCCTTCTAAAAAAAAAAAAAAAAAAAA
```

**Figure 3.** The nucleotide and deduced amino acid sequence of the pBFSTX insert. The region of the gene cloned from the genomic DNA by PCR reactions is underlined. Numbers indicate amino acid residues. Start and stop codons are underlined and in bold.

```
          1                                                  50
pBFSTX                MEEILKTLPQ HTCSWLKHKI IMYKYQDFWT SKQLLEGTLM
pBFST3                --D-I----- ----F---RF TL---K-A-N HQEF---RIL
pFST3                 --D-I----- ----F--QRF TL-----V-N HQEF---RML
pFST4'    METTKTQFES -A-MI-K--- ----S--GR- TL-------G LQNNI--AIL

          51                                                 100
pBFSTX    AQQSFKAEPS DVFLCSAPKT GTTWLKALAF AIVTRENFDE STSPLLKKLV
pBFST3    SE-K---H-N ----A-Y--S --------♦♦I C-I---K--D ------TTMP
pFST3     SE-T---H-N ----A-Y--S ---------- --I---K--D ------TTMP
pFST4'    -------R-D ------Y--S ---------Y ------K--- F-----TNIP

          101                                                150
pBFSTX    HECVPFLERQ VEEIEHNRES SSLPLVATHL PYASLPESVI ASNCRMVYIY
pBFST3    -D-I-L--KD L-K-QE-QRN -LYTPIS--F H-K-----AR T---KI----
pFST3     -D-I-L--KD L-K-QE-QRN -LYTPIS--F H-K-----AR T---KI----
pFST4'    -N-I-YI-KD L-K-VE-QNN -CFTPM---M --HV--K-IL -L--K-----

          151                                                200
pBFSTX    RNIKDVIISN YHFLREAFKL SMEDAPFEET FEDFYNGNSS YGPYWDHILG
pBFST3    --M----V-Y -----QIV-- -V-E-----A -DE-CQ-I-- C----E--K-
pFST3     --M----V-Y -----QIV-- -V-E-----A VDE-CQ-I-- C----E--L-
pFST4'    -------V-F ---G--IT-- PL-D-----A -DE--H-I-Q F------LL-

          201                                                250
pBFSTX    YRKASLDMPD KILFLKYEDL KSEPISNVKR LAEFIGYPFS NDEEKAGVIE
pBFST3    -W----EK-E IF-------M -KD-VPS--K --D---H--T PK--E-----
pFST3     -W----EK-E IF-------M -KD-VPS--K --D---H--T PK--E-----
pFST4'    -W----ER-E V--------V -KD-T----- ---------T FE---E----

          251                                                300
pBFSTX    NIINMCSFEN LSSLEVNKT♦ ♦RKPKGGM♦L ENRLYYRKGQ DGDWKNYFTN
pBFST3    D-VKL----K --------SG MHR-EEAHSI ----F---K ---------D
pFST3     ---KL----K --------SG MHR-EEAHSI -----F---K ---------D
pFST4'    S--KL----- --N-----SG ♦♦NS--FLPI -----F--AK ---------D

          301                  322
pBFSTX    EMKEKIDKIM DEKLSGTGLI LK
pBFST3    --TQ-----I ----GA---V --
pFST3     --I------I ----GA---V --
pFST4'    --T------I -----A---V --
```

**Figure 4.** The amino acid alignment of a putative flavonol sulfotransferase of F. bidentis (pBFSTX) with F. bidentis F3-ST (pBFST3), and F. chloraefolia F3-ST (pFST3) and F4'-ST (pFST4'). Identical amino acids are indicated by dashes (-). Gaps introduced to obtain maximum similarity are shown by diamonds (♦), and numbers represent amino acid residues.

**Figure 5.** Western blot analysis of lysates from E. coli XL1-Blue strain carrying pBluescript SK$^-$ (lanes a, b); pBluescript with BFSTX (lanes c, d); pGEX-3X with BFSTX (lanes e, f); pTrc-His with BFSTX (lanes g, h). Samples in lanes a, c, e and g are lysates of non-induced cells, and those in lane b, d, f and h are those of IPTG-induced cells. Blots were developed using a polyclonal antibody raised against F. chloraefolia F3-ST as a primary antibody. The arrows indicate immunoreactive bands migrating with an estimated molecular weight of 36 kD in lanes d, and h and 58 kD in lane f.

*5*

**Figure 6.** Northern blot analysis of poly(A)$^+$ RNA from F. bidentis terminal buds and cell cultures. One $\mu$g of poly(A)$^+$ RNA from two-day-old cell cultures and terminal buds are shown in lanes a and b, respectively. (A) Blot was probed with [$^{32}$-P]-DNA probe, prepared from the cDNA insert of pBFSTX. The BFSTX transcript of 1.2 kb is indicated by arrow. (B) Blot was probed with [$^{32}$-P]-DNA probe, prepared from the cDNA insert of pBFST3 (B). The BFST3 transcript was detected at an estimated size of 1.3 kb (indicated by arrow).

*6*

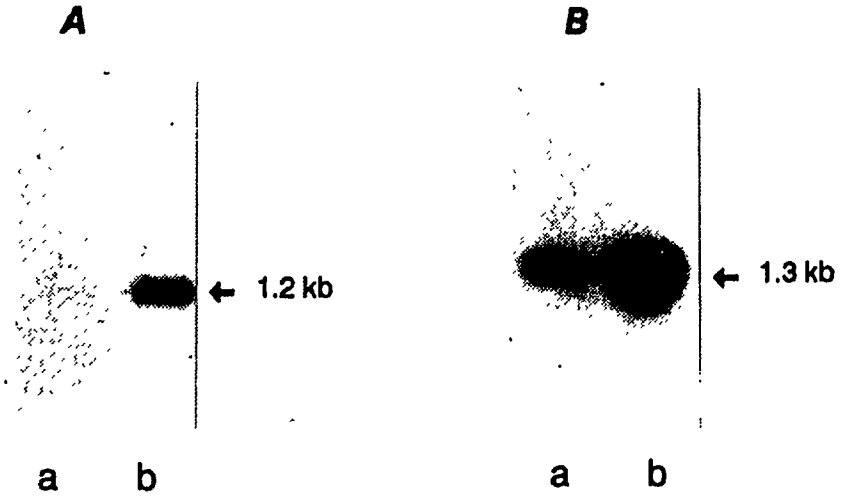
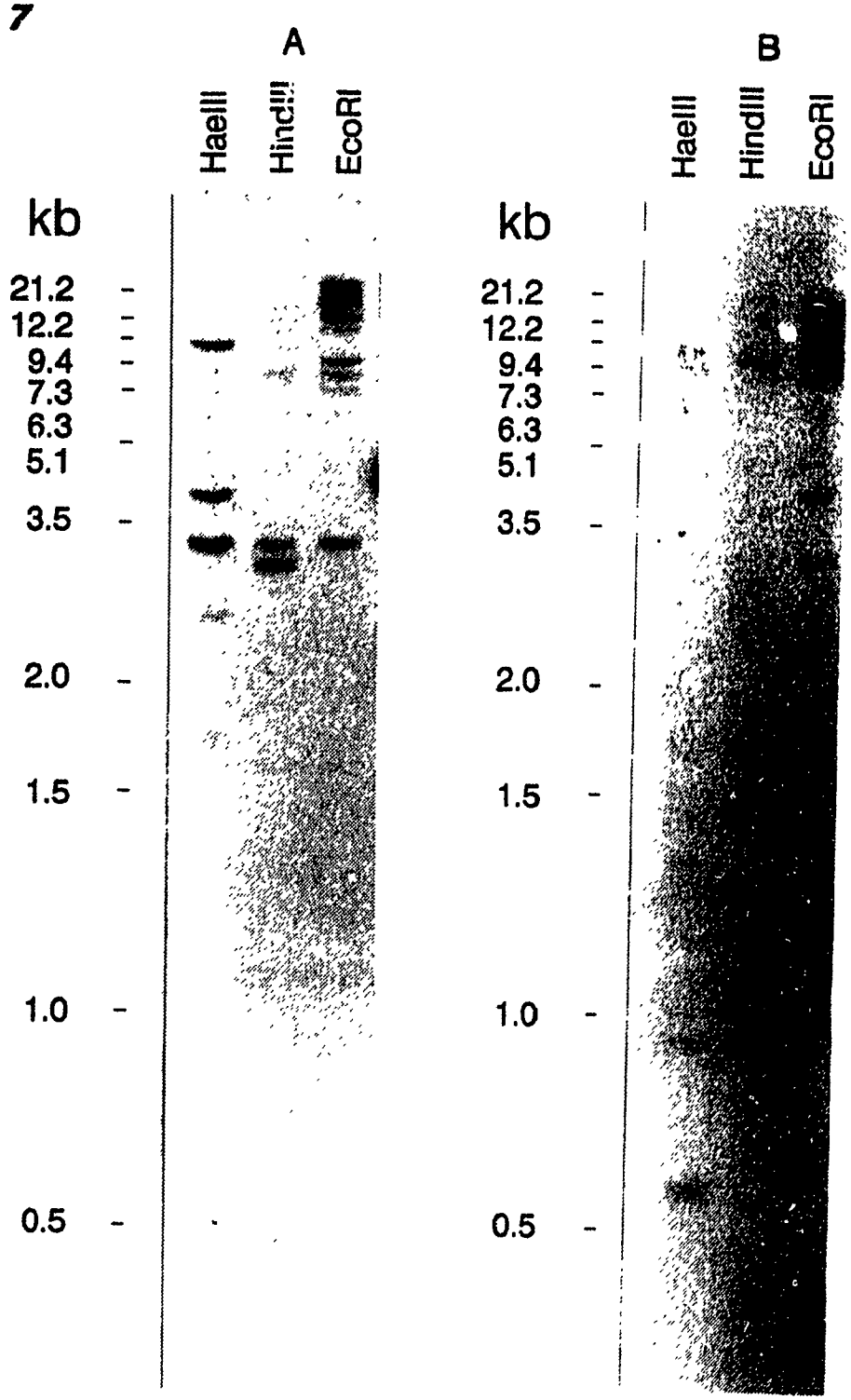


**Figure 7.** Southern blot analysis. Genomic DNA was digested with HaeIII, HindIII and EcoRI. Blots were probed with the cDNA inserts of pBFSTX (A) and pBFST3 (B). Numbers on the left indicate DNA fragment sizes (kb).

7


A
HaeIII
HindIII
EcoRI
kb
21.2
12.2
9.4
7.3
6.3
5.1
3.5
2.0
1.5
1.0
0.5
B
HaeIII
HindIII
EcoRI
kb
21.2
12.2
9.4
7.3
6.3
5.1
3.5
2.0
1.5
1.0
0.5

## CHAPTER V

# Molecular and Biochemical Characterization of Two Nucleoside Diphosphate Kinase cDNA Clones from Flaveria bidentis.


## A. Abstract

Two nucleoside diphosphate kinase cDNA clones have been isolated from Flaveria bidentis by screening an expression library with a polyclonal antibody raised against F. chloraefolia flavonol 3-sulfotransferase (F3-ST). The nucleotide sequences of the two cDNA clones show a high degree of sequence similarity to other reported nucleoside diphosphate kinases (NDPKs), including the putative human tumor suppressor gene NM23 (Gilles et al., 1991) and Drosophila *AWD* regulatory protein (Biggs et al., 1990). When these cDNA clones were expressed in E. coli, their gene products exhibited NDPK enzymatic activity. The recognition of the clones with the antibody raised against the F3-ST suggests a common immuno-epitope and a possible nucleotide binding site of the two proteins.


## B. Introduction

Nucleoside diphosphate kinase (NDPKs, EC. 2.7.4.6.) catalyses the transfer of the terminal phosphate group of nucleoside triphosphates (NTP) to nucleoside diphosphates (NDP). The enzyme is ubiquitous among eukaryotes and shows

little specificity towards the nucleotide bases (Jong and Ma, 1991). The NDPK was reported to catalyze high rate of phosphate transfer (Gilles et al., 1991) and plays a role in maintaining the intracellular NTP pool. Recently, the Drosophila *awd* (Biggs et al., 1990) and human *nm23* gene products (Gilles et al., 1991) which have NDPK enzyme activities have drawn attention to the regulatory function of some NDPKs. The Drosophila *awd* gene is essential for the formation of wing discs and larval development; the mutation of this gene in flies carrying the *prune* eye mutation is lethal, and thus has been named *killer of prune* (*K-pn*) (Biggs et al., 1988). The human *nm23* gene product has been reported to suppress tumor metastasis in mice (Leone et al., 1991). Since *awd* and *nm23* genes are involved in cell developmental processes, their NDPK biochemical function which is involved in the production of GTP led to a proposed role in the regulation of GTP-binding proteins (reviewed by Lacombe and Jakobs, 1992). The NDPK enzyme has been shown to activate the regulatory GTP-binding proteins (Randazzo et al., 1992). However, it has also been shown that a recombinant NM23 protein in which the kinase activity has been eliminated still contained its tumor suppression capability (MacDonald et al., 1993). Furthermore, it has recently been shown that the *nm23* gene product has DNA binding ability and can regulate in vitro transcription even when its NDPK enzyme activity has been eliminated by a site-directed mutagenesis (Postel et al.,

1994).

The autophosphorylation of NDPK has been shown to require the presence of histidine (HIS) at position 122 (Moréra et al., 1994; Dumas et al., 1992). The three dimensional structure of Dictyostelium-NDPK has been investigated, and the ADP binding domain of NDPK has been located at a turn of a distorted helix between ß2 and α2, between the amino acid residues 51-59 (Moréra et al., 1994; Dumas et al., 1992). The latter domain is quite distinct from a classical mononucleotide binding domain which consists of GXXGXXXG, or α/ß motif (Hanks et al., 1988).

Although intensive studies of NDPK have been carried out in Dictyostelium, human, Drosophila and yeast systems with respect to secondary structures and regulatory functions, very little is known about a possible regulatory role of NDPKs in plants. NDPKs have recently been purified and cloned from spinach (Nomura et al., 1991; Nomura et al., 1992; Zhang et al., 1993), rice (Yano et al., 1993), Arabidopsis (Quigley, 1992), and Pisum sativum (Finan et al., 1994). In this report, the isolation of two cDNA clones encoding NDPK from Flaveria bidentis is described. These clones were obtained by screening of a cDNA expression library using a polyclonal antibody raised against F. chloraefolia flavonol 3-sulfotransferase (F3-ST). The putative common epitope between NDPK and sulfotransferases is discussed.

## C. Materials and Method

### C.1 Plant Material

Seeds of Flaveria bidentis var. angustifolia O.K. were kindly provided by Dr. H.R. Juliani, University of Cordoba, Argentina. Seeds were germinated in vermiculite on top of potting soil and further propagated by cuttings. A callus culture of F. bidentis was initiated from leaf disks and maintained on a MS semi-solid medium containing 3% w/v sucrose, 4.5 $\mu$M 2,4-D and 0.45 $\mu$M kinetin (Bleichert et al., 1989). Calli were broken into small pieces and transferred to a liquid medium of the same composition. The suspension culture was maintained in the light, at room temperature, in 1-L nipple flasks rotating centripetally at 4 rpm.

### C.2 Chemicals

The immunodetection kit was from Bio-Rad (Mississauga, ONT); the UniZap XR cDNA synthesis kit from Stratagene (La Jolla, CA), and the Sequenase Version 1.0 sequencing kit from USB (Cleveland, OH). Restriction endonucleases and DNA modifying enzymes were from BioCan (Mississauga, ONT). All other chemicals were analytical grade.

### C.3 F. bidentis cDNA library construction and immunoscreening

Total RNA was isolated by the method of Logeman et al (1987), and poly(A)+ RNA was purified by chromatography on

oligo(dT)-cellulose (Aviv and Leder, 1972). A cDNA library was constructed in lambda UniZap XR according to the manufacturer's instructions. The cDNA library was screened by immunodetection as described in Sambrook et al. (1989) with anti-flavonol 3-ST immune serum (1:500) as primary antibody. The alkaline phosphatase-conjugated anti-rabbit IgG antibody was used as the secondary antibody according to manufacturer's instruction.

**C.4 Southern blot analysis**

DNA was fractionated by gel electrophoresis through 1% w/v agarose gel using Tris-borate electrophoresis buffer (pH 8.5). The fractionated DNAs were visualized under UV illumination following ethidium bromide staining. The gel was placed in the denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 min at room temperature, then transferred to a neutralizing solution (0.5 M Tris-HCl, pH 7.2, 1.5 M NaCl, 1 mM EDTA) twice for 15 min. The fractionated DNAs were transferred to Hybond-N nylon membrane (Amersham) by capillary blotting (Sambrook et al., 1989), and fixed to the membrane by baking at 80°C for 2 h. Prehybridization and hybridization for Southern blots were performed as described by Gulick and Dvorak (1990). The blots were then washed 3 times with a solution containing 0.1x SSC, 1% w/v SDS and 0.1% w/v sodium pyrophosphate, for 20 min at 55°C. The blots were exposed to Fiji RX film with an intensifing screen at -80°C for various periods of time. The

sizes of DNA fragments were estimated by comparison with molecular weight markers: either 1 kb ladder (Gibco BRL), or HindIII-EcoRI digested lambda DNA markers (BioCan).

**C.5 Northern blot analysis**

RNA was isolated from terminal bud tissue and from cultured cell suspension of F. bidentis two days after subculturing by the method of Logemann et al (1987), and the poly(A)+ RNA was purified by chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972). One $\mu$g of poly $(A)^+$ RNA was fractionated on 1% agarose formaldehyde gel using an electrophoresis buffer containing 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0. The fractionated RNA was transferred onto Hybond-N nylon membrane (Amersham), and fixed on to the membrane by UV crosslinking. Prehybridization and hybridization for northern blots were performed as described by Gulick and Dvorak (1990). Following hybridization, the membranes were washed 3 times with a solution containing 0.1x SSC, 1% w/v SDS and 0.1% w/v sodium pyrophosphate, for 20 min at 55°C. The blots were exposed to Fiji RX film at -80°C for 4 to 24 h. The sizes of the RNAs were estimated by comparson with RNA molecular weight markers (Gibco BRL).

**C.6 DNA probe preparation**

Twenty five ng of cDNA inserts from XbaI, XhoI digested pNDPK-a, or pNDPK-b were used for a random hexanucleotide

primer elongation reaction in the presence of [$\alpha$-$^{32}$P] dCTP (50 $\mu$Ci) using the Amersham random priming kit. The labelled fragments were then passed through a 1-mL Sephadex G-50 column to remove unincorporated nucleotides.

**C.7 DNA sequencing**

A series of cDNA subclones pNDPK-a and pNDPK-b were generated using internal MboI restriction sites. Clones were sequenced in both directions using the Sequenase version 1 kit according to manufacturer's instructions. Analysis of nucleotide sequences and the derived amino acid sequences were performed using the GCG software, version 7 (Genetics Computer Group, 1991).

**C.8 NDPK expression in *E. coli***

The cDNA inserts of pNDPK-a and pNDPK-b had a very short 5'-UTRs and the apparent ATG start codons are in-frame with ATG start codon of the lacZ gene of pBluescipt SK$^-$. These clones were used for the expression of the gene products. Bacteria strain XL1-Blue, containing pNDPK-a and pNDPK-b, were grown overnight at 37°C in LB medium with 100 $\mu$g.mL$^{-1}$ tetracycline, 50 $\mu$g.mL$^{-1}$, and 100 $\mu$l of overnight culture was used to innoculate 3 mL of the same media at 37°C. IPTG was added after 1 h to a final concentration of 1 mM. After an additional 2 h, the culture was centrifuged (10,000xg), and the cell pellets were resuspended in 1 mL of the enzyme assay

buffer (50 mM Tris-HCl, pH 7.5, 14 mM 2-ME), then lysed by sonication. The lysate was cleared by centrifugation (10,000xg) and the supernatant was desalted by passage through a PD-10 column (Pharmacia). Protein concentrations were determined according to the method of Bradford (1976), using the Bio-Rad reagent and BSA as the protein standard. The desalted protein preparations were used as the source for NDPK assay. Control cultures contained XL1-Blue cells harboring the pBluescript $SK^-$ plasmid.

The method of Koyama (1984) was employed to determine the formation of phosphoenzyme intermediates. The reaction mixture (100 $\mu$L) contained 20 mM Tris-HCl (pH 7.5), 4 mM DTT, 1mM EDTA, 0.6 M KCl, 1mM $MgCl_2$, 2 $\mu$M [$\gamma$-$^{32}$P]ATP (650 cpm/pmol) and various amounts of protein extracts. The mixture was incubated on ice for 15 min, and the reaction was stopped by the addition of 1 mL of 0.1 M sodium pyrophosphate containing 10 mM EDTA. The mixture was immediately passed through a nitrocellulose membrane (Bio-Rad) and washed 3 times with sodium pyrophosphate buffer. Following membrane drying, the radioactivity of the membrane was measured by liquid scintillation counting. For the inhibition of [$\gamma$-$^{32}$P]-phosphoenzyme formation by the anti-F3-ST antibody, the reaction mixture was incubated on ice with the antibody, diluted at 1:500 to 1:3000, for 30 min prior to the addition of [$\gamma$-$^{32}$P]ATP.

### C.9 Western blot analysis

Protein extracts (10 $\mu$g) were fractionated by SDS-PAGE using 12% acrylamide gels. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes using a semi-dry electrotransfer apparatus according to the manufacturer's instructions (Bio-Rad). The blots were developed with anti-flavonol 3-ST immune serum (1:2000) or nonimmune serum (1:2000) as primary antibody, then with alkaline phosphatase-conjugated anti-rabbit IgGs as the secondary antibody, according to manufacturer's instruction (Bio-Rad).

## D. Results

### D.1 Characterization of nucleoside diphosphate kinase cDNA clones (pNDPK-a and pNDPK-b)

A F. bidentis cDNA expression library was screened with a polyclonal antibody raised against F. chloraefolia F3-ST (Varin and Ibrahim, 1992). Eleven cDNA clones that reacted with the antibody exhibited high sequence similarities to NDPKs; preliminary sequencing indicated that the clones were derived from two distinct genes, referred to here as NDPK-a, and NDPK-b. Full length DNA sequences showed that the clones have inserts of 620 and 592 bp respectively. The open reading frames (ORF) of both NDPK-a and NDPK-b start at nucleotide 24 and terminate at nucleotide 452 (Fig. 1).

Both pNDPK-a and pNDPK-b cDNA clones were expressed in E. coli, and the lysates of the IPTG-induced bacteria containing the plasmids were obtained and assayed using the method of Koyama et al (1984) for the formation of phosphoenzyme intermediate ([$\gamma$-$^{32}$P]phosphoenzyme). Radioactivity values of 3,000 ± 50 and 6,000 ± 100 dpm/$\mu$g protein (average values from 3 independent assays) were obtained from cultures containing pNDPK-a and pNDPK-b, respectively; whereas the value for the control was 60 dpm/$\mu$g protein. When the lysates were incubated with the antibody on ice for 30 min prior to enzyme assays, the radioactivities decreased to 1,000 ± 57 and 1,200 ± 120 dpm/$\mu$g protein for NDPK-a and NDPK-b, respectively. The values obtained from pBluescript SK$^-$ control cells showed no effect after antibody treatment (Table 1).

Western blot analysis of IPTG-treated E. coli cultures containing pNDPK-a and pNDPK-b indicated that the induced gene products cross-reacted with the polyclonal antibody raised against F3-ST (Fig. 2). The molecular weight of the gene products was 20 kD which corresponds to the molecular weight predicted from the cDNA coding sequence plus 43 amino acid residues encoded by the vector sequence and the 5'-UTR of the clones.

**D.2 Sequence analysis of pNDPK-a and pNDPK-b**

Both pNDPK-a and pNDPK-b cDNA clones share an overall 88.3% nucleotide sequence similarity, and 92.2% nucleotide

sequence similarity within their ORFs. The deduced amino acid sequences of pNDPK-a and pNDPK-b ORFs, contain 148 amino acid residues with predicted molecular weights of 16,136 and 16,200 respectively. The two amino acid sequences show 96.6% similarity (Fig. 3).

The deduced amino acid sequences of NDPK-a and NDPK-b show respectively 76.9% and 79.6% similarity to human *nm23*, referred to as Hsnm23 in Fig. 3, (Gilles et al., 1991); 79.1% and 80.4% similarity to Drosophila *awd*, awdr, (Biggs et al., 1990); 72.3% and 73.6% similarity to Dictyostelium NDPK, Ddindk, (Troll et al., 1993); 91.8% and 93.9% similarity to Pisum sativum-NDPK, ndk-p1 (Finan et al., 1994) (Fig. 3).

Since both pNDPK-a and pNDPK-b were isolated using an anti-F3-ST antibody, the deduced amino acid sequences of NDPK-a and NDPK-b were compared to that of F3-ST from F. bidentis using the BESTFIT, COMPARE and DOTPLOT programs (Genetics Computer Group, 1991). The entire amino acid sequence of NDPK-a and NDPK-b revealed 47.9% and 48.6% similarity, respectively to F. bidentis F3-ST (pBFST3). However, the amino acid residues 46-64 of NDPK-a and NDPK-b exhibit 73.7% and 68.4% similarity, respectively to the amino acid residues 172-191 of pBFST3 (Fig. 4).

**D.3 Northern and Southern blot analysis**

Northern blots probed with the insert of pNDPK-b showed a 800 bp transcript present in samples prepared from both F.

bidentis terminal buds and cell suspension culures (Fig. 5). Genomic DNA was digested with restriction endonucleases HaeIII, HindIII, and EcoRI, and hybridized to a probe prepared from pNDPK-b. The probe detected two bands, migrating at estimated molecular weights of 2.4 and 1.85 kb in HaeIII-digested DNA, and of 3.2 and 1.3 kb in HindIII-digested DNA and detected three bands migrating at estimated molecular weights of 9.6, 7.0 and 6.2 kb in EcoRI-digested DNA (Fig. 6). At least two NDPK gene members are apparently present in the F. bidentis genome.

**E. Discussion**

We report the isolation of two cDNA clones (pNDPK-a and pNDPK-b). Both cDNA clones show high sequence similarity to other reported NDPKs (Fig. 3). Dictyostelium-NDPK is the most documented NDPK, in terms of its function as well as its primary and tertiary protein structures. The entire amino acid sequence of Dictyostelium-NDPK, (referred to as Ddindk in Fig. 3) exihibits 72.3% and 73.6% similarity to NDPK-a and NDPK-b, respectively (Troll et el., 1993). In addition, the Dictyostelium-NDPK amino acid residues between 8 and 138, which are important for the formation of globular α/ß domains, exhibit 77.1% and 78.6% similarity to the equivalent amino acid residues of NDPK-a and NDPK-b, respectively (Dumas et al., 1992; Moréra et al., 1994). This high sequence similarity between Dictyostelium-NDPK and F. bidentis-NDPKs

suggests that their three-dimensional structures are quite similar. The NDPK-a and NDPK-b proteins contain $HIS^{115}$, equivalent to $HIS^{122}$ of Dictyostelium-NDPK which is a critical amino acid for the catalytic activity; $PRO^{93}$, equivalent to $PRO^{100}$ of Dictyostelium-NDPK which is important for the formation of polymerized enzyme form; $SER^{117}$, equivalent to $SER^{124}$ Dictyostelium-NDPK which may be important for the regulatory function of NDPK (Dumas et al., 1992; Moréra et al., 1994); MacDonald et al., 1993). In addition, NDPK-a and NDPK-b contain a significant domain for the nucleoside diphosphate binding site, and the amino acid residues 44-52 which are equivalent to residues 51-59 of Dictyostelium-NDPK (Moréra et al., 1994). Moreover, both pNDPK-a and pNDPK-b when expressed in E. coli showed high activity for the formation of [$\gamma$-$^{32}$P]-phophoenzyme intermediate which is typical of NDPK enzymes.

The high amino acid sequence similarities of NDPK-a and NDPK-b to human *nm23* (76.9% and 79.6% similarity, respectively) and to Drosophila *awd* (79.1% and 80.4% similarity, respectively) draw attention to a possible regulatory function for NDK's of the pNDPK-a and pNDPK-b gene products. However, human *nm23* and Drosophila *awd* are the only NDPK genes that have been shown to have regulatory functions. Moreover, MacDonald et al. (1993) have separated regulatory functions and NDPK activity by site-directed mutgenesis of *nm23*. The authors have also shown $SER^{41}$ to be critical for its

regulatory activity in tumor metastatic suppression. Neither pNDPK-a nor pNDPK-b of F. bidentis has a SER or any obvious kinase substrate at this position. No function other than NDPK activity can be infered from data presented here.

The initial selection of both pNDPK-a and pNDPK-b clones by cDNA library screening with a F3-ST specific antibody suggests the existence of common epitopes between these enzymes. This was confirmed by western blot analysis of the pNDPK-a and pNDPK-b gene products expressed in E. coli cultures. The cross reactivity of this antibody is somewhat surprising, since two enzymes, NDPK and F3-ST, have distinct catalytic activities and do not share common substrates. However, the binding sites for the related substrates, ATP and NDP (for the NDPK's) and 3'-phosphoadenosine 5'-phosphosulfate (PAPS, for F3-ST) are likely responsible for the cross reactivity of the antibody. In fact, the amino acid residues 46-64 of NDPK-a and NDPK-b have 73.7% and 68.4% similarity to the amino acid residues 171-191 of the F3-ST clone, pBFST3 (Ananvoranich et al., 1994), as compared to an overall similarity of 47.9 and 48.6% between the coding sequence of pNDPK-a or pNDPK-b to pFBST3, respectively. The amino acid residues 52-59 of Dictyostelium-NDPK, which align to amino acid residues 44-52 of NDPK-a and NDPK-b, were found in Dictyostelium-NDPK to form a loop between ß2 and α2 strands, and would therefore be an accessible domain on the protein surface of the functional NDPK protein. Moreover, this area

contains a nuclectide binding site (Moréra et al., 1994). F3-ST catalyses the formation of flavonol 3-sulfate using flavonols and PAPS as substrates and co-substrate, respectively. The high similarity between F. bidentis pNDPK-a/pNDPK-b and pBFST3 suggests that the amino acid residues 171-191 of pBFST3 might be a binding site for PAPS, whose structure is closely related to NDP and NTP. This region of pBFST3 exhibits similarity to several sulfotransferases isolated from a wide range of aminal and plant speices (Weinshilboum and Otterness, 1994). However, this region is not located in the conserved region IV of the reported sulfotransferases, which consists of GXXGXXXGK and has been proposed to be related to the P-loop of a nucleotide binding site or PAPS binding site (Weinshilboum and Otterness, 1994). Photoaffinity labelling of PAPS to F3-ST protein or site-directed mutageneis of the putative sites in F3-ST would thus provide more evidence to confirm the PAPS binding site.

## F. LITERATURE CITED

Ananvoranich S, Varin L, Gulick P, Ibrahim RK (1994) Cloning and regulation of flavonol 3-sulfotransferase in cell suspension culture of Flaveria bidentis. Plant Physiol, in press

Aviv H, Leder P (1972) Purification of biologically active globin mRNA by chromatography on oligothymidylic acid cellulose. Proc Natl Acad Sci USA 69: 1408-1412

Biggs J, Tripoulas N, Hersperger E, Dearolf C, Shearn A (1988) Analysis of the lethal interaction between the prune and the Killer of prune mutations of Drosophila. Gene Dev 2: 1333-1343

Biggs J, Hersperger E, Steeg PS, Liotta LA, Shearn A (1990) A Drosophila gene that is homologous to a mammalian gene associated with tumor metastasis codes for a nucleoside diphosphate kinase. Cell 63: 933-940

Bleichart E, Varin L, Hannoufa A, Ibrahim RK (1989) In vitro culture of Flaveria spp. and its potential for the biosynthesis of sulfated flavonoids. Plant Physiol 89: S-187

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72: 248-254

Dumas C, Lascu I, Moréra S, Glaser P, Fourme R, Wallet V, Lacombe M, Véron M, Janin J (1992) X-ray structure of nucleoside diphosphate kinase. EMBO J 11: 3203-3208

Finan PM, White IR, Redpath SH, Findlay JBC, Millner PA (1994) Molecular cloning and sequence determination and heterologous expression of nucleoside diphosphate kinase from Pisum sativum. Plant Mol Biol 25: 59-67

Genetics Computer Group (1991) Program manual for the GCG package, Version 7, April 1991, 575 Science Drive, Madison, WI

Gilles AM, Presecan E, Vonica A, Lascu I (1991) Nucleoside diphosphate kinase from human erythrocytes. J Biol Chem 266: 8784-8789

Gulick PJ, Dvorak J (1990) Selective enrichment of cDNAs from salt-stress-induced genes in the wheatgrass Lophopyrum elongatum by the formamide-phenol emulsion reassociation technique. Gene 95: 173-177

Hanks S, Quinn AM, Hunter T (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241: 42-52

Jong A, Ma J (1991) Saccharomyces cerevisiae nucleoside diphosphate kinase: purification, characterization, and substrate specificity. Arch Biochem Biophys 291: 241-246

Koyama K, Yokoyama M, Koike T, Ohtsuki K, Is...da N (1984) Nucleoside diphosphate kinase from Ehrlich ascites tumor cells. J Biochem 95: 925-935

Lacombe ML, Jakobs KH (1992) Nucleoside diphosphate kinase as potential new targets for control of development and cancer. Trends Pharmacol Sci 13: 46-48

Leone A, Flatow U, Richter-King C, Sanden M, Margoulies IMK, Liotta LA, Steeg PS (1992) Reduced tumor incidence, metastatic potential and cytokine responsiveness of nm23-transfected melanoma cells. Cell 65: 25-35

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissue. Anal Biochem 163: 16-20

MacDonald NJ, De la Rosa A, Benedict MA, Freije JMP, Krutsch H, Steeg P (1993) A serine phosphorylation of NM23, and not its nucleoside diphosphate kinase activity, correlates with suppression of tumor metastatic potential. J Biol Chem 268: 25780-25789

Moréra S, Lascu I, Dumas C, LeBras G, Briozzo P, Véron M, Janin J (1994) Adenosine 5'-diphosphate binding and the active site of nucleoside diphosphate kinase. Biochemistry 33: 459-467

Nomura T, Yatsunami K, Handa A, Sugimoto Y, Fukui T, Zhang J, Yamamoto J, Ichikawa A (1992) The amino

acid sequence of nucleoside diphosphate kinase I from spinach leaves, as deduced from the cDNA sequence. Arch Biochem Biophys **297**: 42-45

Nomura T, Fukui T, Ichikawa A (1991) Purification and characterization of nucleoside diphosphate kinase from spinach leaves. Biochim Biophys Acta **1077**: 47-55

Postel EH, Ferrone CA (1994) Nucleoside diphosphate kinase enzyme activity of NM23-H2/PuF is not required for its DNA binding and in vitro transcriptional functions. J Biol Chem **269**: 8627-8630

Quigley FR (1993) Arabidopsis thaliana gene for nucleoside diphosphate kinase. EMBL database accession number X69376 ID ATNDK 13 AC

Randazzo PA, Northup JK, Kahn RA (1992) Regulatory GTP-binding proteins (ADP-ribosylation factor, $G_t$ and RAS) are not activated directly by nucleoside diphosphate kinase. J Biol Chem **267**: 18182-18189

Sambrook J, Fritsch, EF, Maniatis T (1989) Molecular cloning, a laboratory manual. Second edition, Cold Spring Harbor Laboratory Press, New York

Troll H, Winckler T, Lascu I, Müller N, Saurin W, Véron M, Mutzel R (1993) Separate nuclear genes encode cytosolic and mitochondrial nucleoside diphosphate kinase in Dictyostelium discoideum J Biol Chem **268**: 25469-25475

Varin L, Ibrahim RK (1992) Novel flavonol 3-sulfotransferase: purification, kinetic properties, and amino acid sequence. J Biol Chem **267**: 1858-1863

Weinshiboum R, Otterness D (1994) Sulfotransferase enzymes. In FC Kauffman, ed, Conjugation-Deconjugation Reactions in Drug Metabolism and Toxicity; Handbook of Experimental Pharmacology, Vol 112. Springer-Verlag, London, pp 45-78

Yano A, Shimazaki T, Kato A, Umeda M, Uchimiya H (1993) Molecular cloning and nucleotide sequence cDNA encoding nucleoside diphosphate kinase of rice (Oryza sativa L.). Plant Mol Biol **23**: 1087-1090

Zhang J, Nomura T, Yatsunami K, Honda A, Sugimoto Y, Moriwaki T, Yamamoto J, Ohta M, Fukui T, Ichikawa A (1993) Nucleotide sequence of the cDNA encoding nucleoside diphosphate kinase II from spinach leaves. Biochim Biophys Acta **1171**: 304-306

**Table 1.** The phosphate-incorporating activity of pNDPK-a and pNDPK-b.

| Treatment | dpm/μg protein | | |
|---|---|---|---|
| | NDPK-a | NDPK-b | Control |
| [γ-$^{32}$P]ATP | 3,000 ± 50[1] | 6,000 ± 100 | 60 ± 5 |
| Incubation with antibody prior to addition of [γ-$^{32}$P]ATP | 1,000 ± 57 | 1,200 + 120 | 60 ± 15 |

The pNDPK-a and pNDPK-b were expressed in E. coli XL1-Blue strain. The NDPK enzyme assays monitored the radioactivity of [γ-$^{32}$P]-phosphoenzyme in lysates. Values are averages of three independent assays. Control values are from control cultures containing the pBluescript SK$^-$ plasmid without insert. Antibody treatment consisted of 30-min incubation with anti-F3-ST immune sera, diluted at 1:3000, prior to addition of [γ-$^{32}$P]ATP to the assay.

[1]Standard error of mean.

**A**

```
      CAAAGATGGAGCAAACCTTTATCATGATCAAACCCGATGGCGTTCAAAGCGGCCTCGTT
1           M  E  Q  T  F  I  M  I  K  P  D  G  V  Q  S  G  L  V
      GGTGAAATCATTGGTAGATTTGAGAAGAAAGGCTTCTCTTTGAAAGGGTTGAAGCTTTTG
19     G  E  I  I  G  R  F  E  K  K  G  F  S  L  K  G  L  K  L  L
      ACTGTTGACCGAGCTTTTGCTGAGAAGCATTATGCTGATTTGTCCTCAAAGCCTTTCTTC
39     T  V  D  R  A  F  A  E  K  H  Y  A  D  L  S  S  K  P  F  F
      AACGGTCTAGTTGAGTACATCGTCTCTGGTCCCGTTGTTGCCATGGTTTGGGAGGGCAAG
59     N  G  L  V  E  Y  I  V  S  G  P  V  V  A  M  V  W  E  G  K
      AATGTGGTCACCACTGGCCGCGAGATCATCGGTGCTACAAACCCTGCTGAGTCTGCCCCT
79     N  V  V  T  T  G  R  E  I  I  G  A  T  N  P  A  E  S  A  P
      GGTACCATCCGTGGTGATTTTGCAATCGACATTGGCAGAAATGTGATTCATGGTAGTGAT
99     G  T  I  R  G  D  F  A  I  D  I  G  R  N  V  I  H  G  S  D
      GCGGTTGAGAGCGCGAGGAAGGTGATCGGTCTGTGGTTCCCCGAGGGTGTTGCCAATTGG
119    A  V  E  S  A  R  K  V  I  G  L  W  F  P  E  G  V  A  N  W
      TCAAGCAGCCTTCACCCATGGATCTATGAATGAGTGTGATTTAAGCCTCATGTTATTATG
139    S  S  S  L  H  P  W  I  Y  E  -
      ATTATCACTCTTGACTAGTATGCTTTGTGGGTGTTATCTACTTTCAGTTGGTTTTGTTTA
      CTGATTTTGACATTGCGCGAGGAGCTTCTTTTCGCTGTTTAATCGGTTTTACTATTGTGT
      TAAAAAAAAAAAAAAAAAAA
```

**B**

```
      CAAAGATGGAGCATACCTTTATCATGATCAAGCCTGATGGCGTTCAAAGAGGCCTTGTT
1           M  E  H  T  F  I  M  I  K  P  D  G  V  Q  R  G  L  V
      GGTGAAATCATTGGTAGATTTGAGAAGAAAGGCTTCTCTTTGAAAGGATTGAAGCTTTTA
19     G  E  I  I  G  R  F  E  K  K  G  F  S  L  K  G  L  K  L  L
      ACTGTGGACCAAGCTTTCGCTGAGAAGCACTATGCTGATTTGTCCGCAAAGCCTTTCTTC
39     T  V  D  Q  A  F  A  E  K  H  Y  A  D  L  S  A  K  P  F  F
      AACGGGCTAGTTGAGTACATCATCTCTGGACCCGTTGTTGCCATGGTTTGGGAAGGCAAG
59     N  G  L  V  E  Y  I  I  S  G  P  V  V  A  M  V  W  E  G  K
      AATGTGGTTACCACTGGGCGCAAGATCATTGGTGCCACAAACCCTGCTGAGTCTGCCCCT
79     N  V  V  T  T  G  R  K  I  I  G  A  T  N  P  A  E  S  A  P
      GGCACCATCCGTGGTGATTTTGCAATCGACATTGGCAGAAATGTGATTCATGGTAGTGAT
99     G  T  I  R  G  D  F  A  I  D  I  G  R  N  V  I  H  G  S  D
      GCTGTTGAAAGTGCAAAGAAGGAGATTGCTTTGTGGTTCCCCGAGGGTGTCGCCAACTGG
119    A  V  E  S  A  K  K  E  I  A  L  W  F  P  E  G  V  A  N  W
      TCAAGCAGCCTTCACCCATGGATCTACGAATGAGTGTTGTTTAACCTTTATGGTATGATC
139    S  S  S  L  H  P  W  I  Y  E  -
      TAGTATGATGGTGTGATCTGTTTGAAAACTGATCTTTGTTTATGCATGAGGAATTGTTTT
      GAGAGTTAAATCAGTTTTATTGTCGCATTTGTTAAAAAAAAAAAAAAAAAAAA
```

**Figure 1.** The nucleotide and amino acid sequence of pNDPK-a (A) and pNDPK-b (B). Numbers indicate amino acid residues. Start (ATG) and stop (TGA) codons are underlined and in bold letters.

**Figure 2.** Western blot analysis of E. coli lysates from IPTG-induced E. coli carrying pBluescript SK⁻ (lane a), pNDPK-a (lane b), and pNDPK-b (lane c). The arrow indicates gene products migrating at 20 kD in lane a and b. Other immunodetected bands are resulted from the immuno-cross reactions of E. coli proteins to the antibody and also observed in the controls. Sizes in kD of protein molecular weight markers (Mr) are indicated on the left.

*2*

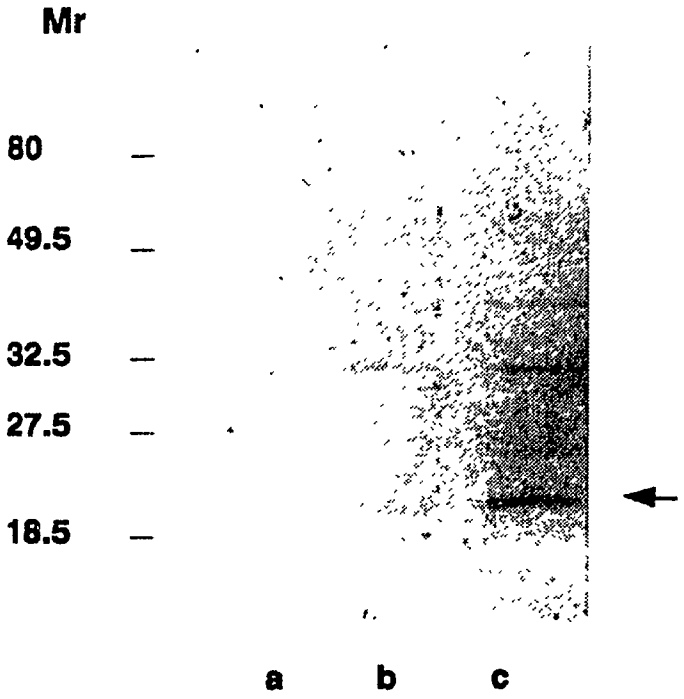


**Figure 3.** Deduced amino acid sequence alignment of two cDNA clones of Flaveria bidentis NDPK-a (GenBank accession number U10282), NDPK-b (U10283) and previously reported NDPK sequences: Dictyostelium discoideum Ddindk (J05457), Ruttus norvegicus ndpk21 (M55331), Drosophila melanogaster Dmawdr (X13107), Homo sapiens Hsnm23 (X73066), Saccharomyces cerevisiae YNK (S64016), Arabidopsis thaliana NDK (X69373), A. thaliana NDK13 (X69376), Spinacia oleracea NDKI (S40546), Pisum sativum ndk-p1 (X71388), Oryza sativa ndk-r (D16292), S. oleracea NDKII (D11465), D. discoideum ndkM (L23068), E. coli Ecndk (X57555). Identical amino acids are shown by dashes (-); gaps introduced to obtain maximum similarity are indicated by diamonds (♦); stop codons are indicated by asterisks (*).

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| NDPK-a | F.bidentis | (1-46) | MEQTFI | MIKPDGVQSG | LVGEIIGRFE | KKGFSLKGLK | LLTVDRAFAE |
| NDPK-b | F.bidentis | (1-46) | --H--- | --------R- | ---------- | ---------- | -----Q---- |
| Ddindk | D.discoideum | (4-53) | NKVNK-R--- | AV-----AR- | ------A-Y- | ----V-V--- | Q-VPTKDL-- |
| ndpk21 | R.norvegicus | (1-49) | MANL-R--- | A-------R- | ------K--- | Q---R-VAM- | F-RASEEHLK |
| awdr | D.melanogaster | (1-50) | MAANK-R--- | -V------R- | ---K--E--- | Q---K-VA-- | FTWASKELL- |
| Hsnm23 | H. sapiens | (1-49) | MANC-R--- | A-------R- | ------K--- | Q---R-V--- | FMQASEDLLK |
| YNK | S.cerevisiae | (1-50) | MSSQT-R--- | AV------R- | --SQ-LS--- | ---YK-VAI- | -VKA-DKLL- |
| NDK | A.thaliana | (1-44) | ---- | --------R- | -I--V-C--- | ----T----- | -IS-E-S--- |
| NDK13 | A.thaliana | (1-45) | M----- | --------R- | --SVKSSAGL | RRRV--♦C-- | -IS-E-S--- |
| NDKI | S.oleracea | (1-46) | M----- | --------R- | ------S--- | -------A-- | FVN---P--- |
| ndk-p1 | P.sativum | (1-47) | MA----- | --------R- | -----S--- | ----Y----- | FVN-E----- |
| ndk-r | O.sativa | (1-46) | M--S-- | --------R- | -I-D--S--- | ----Y-R-M- | FMN-E-S--Q |
| NDKII | S.oleracea | (31-130) | SMEQV-E-Y- | --------R- | ------S--- | ----K-I--- | MYPCPKEL-- |
| ndkM | D.discoideum | (68-117) | PGTNQ-RS-- | A--WNST-RR | -I----A--- | ----K-V-I- | I-VPTPEH-A |
| Ecndk | E.coli | (1-48) | MAI-R--S | I---NAVAKN | VI-N-FA--- | AA--K-V-T- | M-HLTVEQ-R |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| NDK-a | F.bidentis | (47-96) | KHYADLSSKP | FFNGLVEYIV | SGPVVAMVWE | GKNVVTTGRE | IIGATNPAES |
| NDK-b | F.bidentis | (47-96) | -------A-- | ---------I | ---------- | ---------K | ---------- |
| Ddindk | D.discoideum | (54-103) | S---EHKER- | --G---SF-T | --------F- | --G--ASA-L | M--V---LA- |
| ndpk21 | R.norvegicus | (50-99) | Q--I--KDR- | --P---K-MN | ---------- | -L---K---V | ML-E----D- |
| awdr | D.melanogaster | (51-100) | -------AR- | --P---N-MN | -----P---- | -L---K---Q | ML------D- |
| Hsnm23 | H. sapiens | (50-99) | E--V--KDR- | --A---K-MH | ---------- | -L---K---V | ML-E----D- |
| YNK | S.cerevisiae | (51-100) | Q---EHVG-- | --PKM-SFMK | ---IL-T--- | --D--RQ--T | -L-----LG- |
| NDK | A.thaliana | (45-94) | ---E-----S | --S---D--- | -------I-- | -----L---K | --------A- |
| NDK13 | A.thaliana | (46-95) | ---E-----S | --S---D--- | -------I-- | -----L---K | --------A- |
| NDKI | S.oleracea | (47-96) | -------A-- | ---------- | ---------- | --G--A---K | L------LA- |
| ndk-p1 | P.sativum | (48-97) | -------A-- | --S---D--I | -------I-- | ---------K | --------Q- |
| ndk-r | O.sativa | (47-96) | Q------D-- | --P------I | ---------- | --D--A---R | -----R-W-A |
| NDKII | S.oleracea | (131-180) | E--K--KA-S | -YQK-ID--T | -----C-A-- | -VG--ASS-K | L----D-LQA |
| ndkM | D.discoideum | (118-167) | ---E--NK-- | ------KFFS | --A-----F- | --D--R---V | L----V-SQ- |
| Ecndk | E.coli | (49-98) | GF--EHDG-- | --D----FMT | ---I-VS-L- | -E-A-QRH-D | LL------NA |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| NDK-a | F.bidentis | (97-145) | APGTIRGDFA | IDIGRNVIHG | SDAVESARKV | IGLWF♦PEGV | ANWSSSLHPW |
| NDK-b | F.bidentis | (97-145) | ---------- | ---------- | -------K-E | -A---♦---- | ---------- |
| Ddindk | D.discoideum | (104-152) | ---S-----G | V-V---.--- | --S----NRE | -A---K---EL | LT♦EVKPN-N |
| ndpk21 | R.norvegicus | (100-149) | K--------C | -QV---I--- | --S----E-E | -----K--EL | IDYK-CA-D- |
| awdr | D.melanogaster | (101-150) | L--------C | -QV---I--- | -------E-E | -A---NEKEL | VT-TPAAKD- |
| Hsnm23 | H. sapiens | (100-150) | K--------C | -QV---I--- | --S----E-E | -----H--EL | VDYT-CAQNW |
| YNK | S.cerevisiae | (101-150) | ---------G | --L----C-- | --S-D--ERE | -N---KK-EL | VD-E-NQAK- |
| NDK | A.thaliana | (95-143) | E--------- | ---------- | --S------E | -A---♦-D-P | V--Q--V--- |
| NDK13 | A.thaliana | (96-144) | E--------- | ---------- | --S------E | -A---♦-D-P | V--Q--V--- |
| NDKI | S.oleracea | (97-145) | E--------- | ---------- | ----D--T-E | -A---♦-D-- | VH-Q----S- |
| ndk-p1 | P.sativum | (98-146) | E--------- | ---------- | -------N-E | -A---♦---A | ---E----S- |
| ndk-r | O.sativa | (97-145) | ------A-Y- | VEV------- | --S-DNGK-E | -A---♦---L | -E-R-N---- |
| NDKII | S.oleracea | (181-230) | E-------L- | VQT----V-- | --SPDNGKRE | -----KEGEI | CQ-TPAQA-- |
| ndkM | D.discoideum | (168-216) | ------F-LC | IET---I--- | --SN---AHE | -A---KEDEI | ---V-TNP* |
| Ecndk | E.coli | (99-144) | LA----A-Y- | DSLTE-GT-- | --S----ARE | -AYF-GEGE- | CPRTR* |

```
NDK-a     46   EKHYADLSS.KPFFNGLVEY     64
               :. :.::|| |::: :::|
BFST3     172  DEFCQGISSCGPYWEHILGY     191
```

**Figure 4.** The amino acid alignment of a region of high similarity between F. bidentis nucleotide diphosphate kinase (NDPK-a) and flavonol 3-sulfotransferase (BFST3). Numbers indicate the amino acid residues. An identical amino acid is indicated by "|". A similar amino acid showing a comparison value (Genetics Computer Group, 1991) greater than or equal to 0.5 is indicated by ":", and that showing a comparison value greater than or equal to 0.1 is indicated by ".".

5

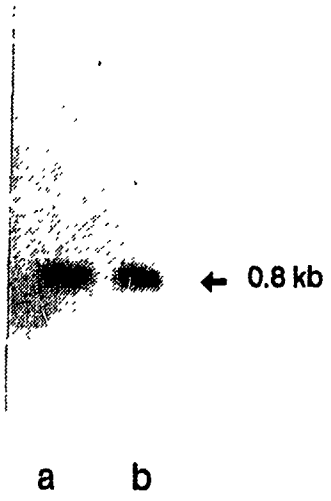
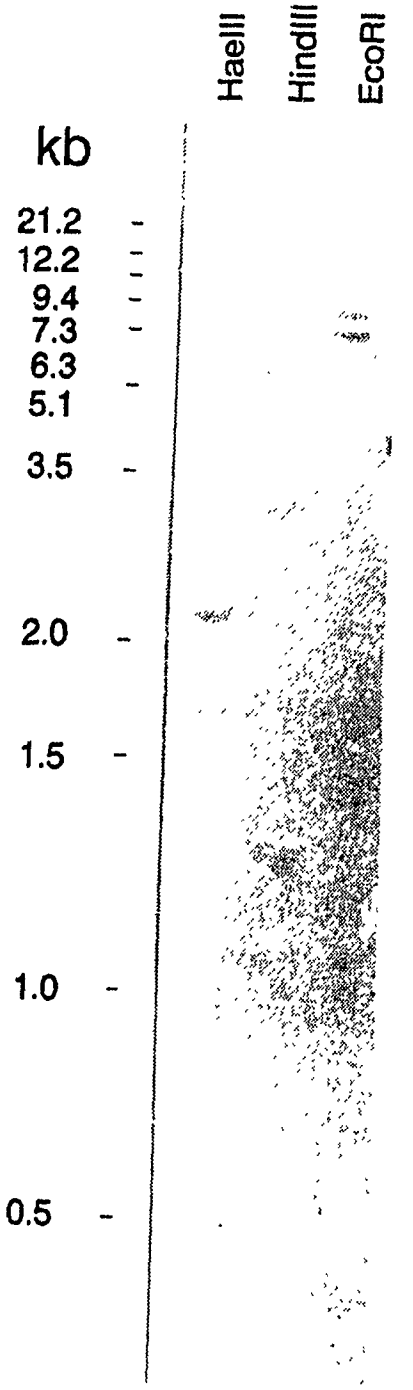



**Figure 5.** Northern blot analysis of NDPK-b. Poly $(A)^+$ RNA from two-day-old subcultures of cell suspension and terminal buds of F. bidentis were in lanes a and b, respectively. A transcript of 800 bp was detected using a [$^{32}$-P]-labeled probe prepared from the cDNA insert of pNDPK-b.

**Figure 6.** Southern blot analysis. Genomic DNA was digested with HaeIII, HindIII, and EcoRI. The DNA fragment sizes of molecular weight markers are indicated on the left. The blot was probed with a [$^{32}$-P]-labeled probe prepared from the cDNA insert of pNDPK-b.

*6*



# CHAPTER VI

## A. Discussion and Conclusions

F. bidentis accumulates a variety of flavonol mono- to tetrasulfates, indicating the presence of a family of F-STs involved in their biosynthesis (Varin, 1992). In contrast with the intact tissues, F. bidentis cell suspension cultures exhibit very low levels of F-ST activity and do not accumulate any flavonol sulfates (Bleichert et al., 1989). The existence of a F3-ST activity in cell suspension cultures was demonstrated, and the culture system was used to study the regulation of F3-ST gene expression. Different compounds, including nutrients, phytohormones, elicitors and flavonols, were tested for their effects on the F3-ST activity of the F. bidentis cultured cells. 2,4-D, a synthetic auxin and flavonol sulfates which exhibited an activating and an inhibitory effect, respectively were further investigated. A cDNA clone (pBFST3) encoding the F3-ST from F. bidentis was isolated, characterized, expressed and used as a probe for the regulation studies. Furthermore, the isolation and characterization of a cDNA clone (pBFSTX) encoding flavonol sulfotransferase-like protein and two cDNA clones (pNDPK-a and pNDPK-b) encoding nucleoside diphosphate kinases were reported.

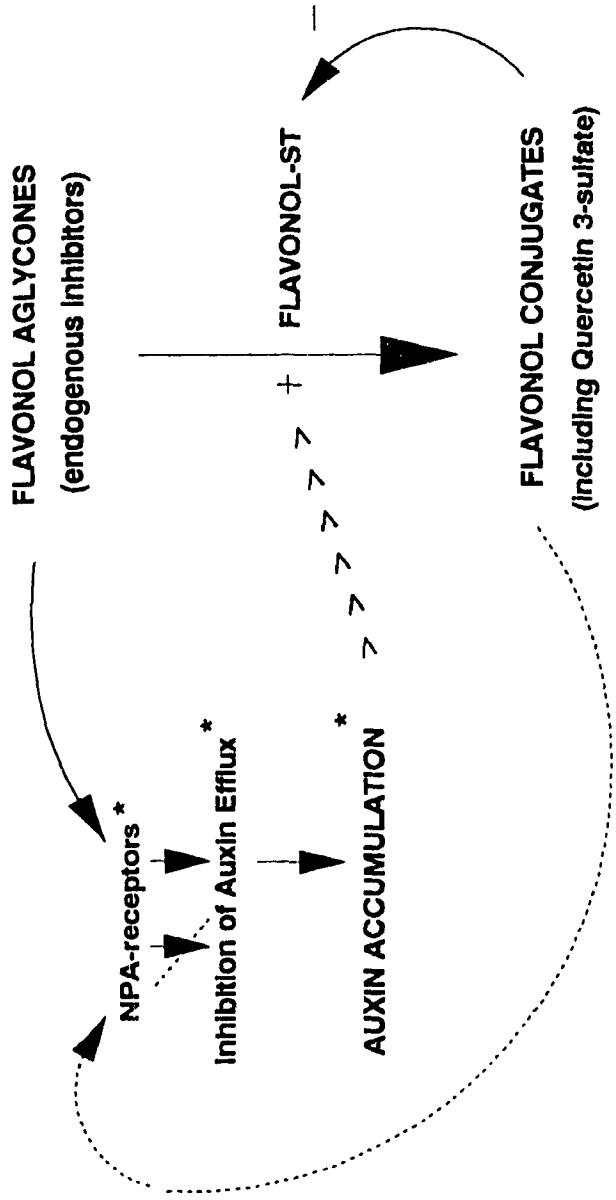
Sequence analysis of the pBFST3 clone revealed high sequence identity with the F3-ST and a moderate level of

identity to the F4'-ST genes from F. chloraefolia, at both the nucleotide and amino acid levels (Varin et al., 1992). Treatment with 2,4-D, which activated F3-ST activity, concomitantly induced F3-ST transcript levels. Pre-incubation of the cultured cells with the transcriptional inhibitor, actinomycin-D reduced the 2,4-D-dependent induction of F3-ST activity. These results suggest that enzyme induction resulting from exposure to 2,4-D is regulated at the level of gene expression. However, exposure of F. bidentis cultures to either quercetin 3-sulfate or quercetin 3,7,4'-trisulfate decreased F3-ST enzyme activity by approximately 50%, but had no effect on F3-ST transcript levels. Quercetin 3-sulfate has been demonstrated to noncompetitively inhibit F3-ST activity in vitro (Varin and Ibrahim, 1992), thus suggesting that the regulation of this enzyme by the sulfated product is not at the transcriptional level, but is likely to be at the enzymatic level. Jacobs and Rubery (1988) reported that some flavonoid aglycones (quercetin, kaempferol or apigenin) bind to the NPA receptor and inhibit the efflux of auxin from the basal end of stem cells, thereby inhibiting polar transport and causing intracellular accumulation. In contrast, flavonol conjugates, including quercetin 3-sulfate, have been shown to strongly bind the NPA receptor and block the quercetin-stimulated accumulation of auxin (Faulkner and Rubery, 1992). The fact that F3-ST gene expression is up-regulated by 2,4-D suggests that the polar transport of auxin may be

autoregulated at the level of gene expression. In such a model (Fig. 1), high levels of auxin would cause increased levels of F3-ST activity and result in the depletion of quercetin and in increased levels of 3-sulfated flavonols. Another point of regulation of auxin transport is the finding that flavonol sulfates inhibit F3-ST activity. Both of these changes would tend to function as a balancing component of the regulation of auxin transport. Therefore, sulfated flavonols may be involved in an interactive mechanism for both the positive and negative control of auxin transport (Fig. 1).

A PCR-mediated cloning strategy was employed to isolate the pBFSTX cDNA clone encoding a flavonol sulfotransferase-like protein. Sequence analysis of the pBFSTX clone revealed high sequence similarity both with the F3-ST from F. bidentis and F. chloraefolia, and the F4'-ST genes from F. chloraefolia at both the nucleotide and amino acid levels (Varin et al., 1992). Although the enzymatic activity of pBFSTX could not be demonstrated using three different E. coli expression vectors (pBluescript SK$^-$, pGEX-3x and pTrc-His) and two yeast expression vectors (pYes-I and pYes-II), western blot analysis using a polyclonal antibody raised against F. chloraefolia F3-ST revealed the nature of the expressed gene products. The failure to detect BFSTX enzymatic activity in the E. coli and yeast expression systems might result from a missing co-factor, a problem of compartmentation, or a protein processing step in E. coli and yeast, as compared with the intact plant

**Figure. 1** A proposed model that sulfated flavonols may be involved in an interactive mechanism for both the positive and negative control of auxin transport. High levels of auxin would cause increased levels of F3-ST activity and result in the depletion of quercetin and in increased levels of 3-sulfated flavonols. Elevated levels of flavonol sulfates would inhibit F3-ST activity resulting in increased level of quercetin. Both of these changes would tend to function as a balancing component of the regulation of auxin transport. *Jacobs and Rubery (1988) reported that some flavonoid aglycones bind to the NPA receptor and inhibit the efflux of auxin causing intracellular accumulation. In contrast, flavonol conjugates, including quercetin 3-sulfate, have been shown to strongly bind the NPA receptor and block the quercetin-stimulated accumulation of auxin (Faulkner and Rubery, 1992).


FLAVONOL AGLYCONES
(endogenous Inhibitors)
FLAVONOL-ST
FLAVONOL CONJUGATES
(including Quercetin 3-sulfate)
NPA-receptors *
Inhibition of Auxin Efflux *
AUXIN ACCUMULATION *

system. In addition, Southern blot analysis using the cDNA inserts of pBFST3 and pBFSTX suggests the presence of at least five members of the flavonol sulfotransferase gene family in the F. bidentis genome.

The isolation and characterization of two cDNA clones (pNDPK-a and pNDPK-b) encoding nucleoside diphosphate kinases were described. Both cDNA clones show high sequence similarity to other reported NDPKs. The initial selection of both pNDPK-a and pNDPK-b clones by cDNA library screening with a F3-ST specific antibody suggests the existence of common immuno-epitopes between these enzymes. This was confirmed by western blot analysis of the pNDPK-a and pNDPK-b gene products expressed in E. coli cultures. The amino acid residues 46-64 of NDPK-a and NDPK-b have 73.7% and 68.4% similarity to the amino acid residues 171-191 of the F3-ST clone (pBFST3), respectively, as compared to an overall similarity of 47.9 and 48.6% between the coding sequence of pNDPK-a or pNDPK-b to pBFST3, respectively. The amino acid residues 52-59 of Dictyostelium-NDPK, which align to amino acid residues 44-52 of NDPK-a and NDPK-b, were found in Dictyostelium-NDPK to form a loop between ß2 and α2 strands and would be an accessible domain on the protein surface of the functional NDPK protein. Moreover, this area contains a nucleotide binding site (Moréra et al., 1994). F3-ST catalyses the formation of flavonol 3-sulfate using flavonols and PAPS as substrates and co-substrate, respectively. The high similarity between F.

bidentis pNDPK-a/pNDPK-b and pBFST3 suggests that the amino acid residues 171-191 of pBFST3 might be a binding site for PAPS, whose structure is closely related to NDP and NTP. This region of pBFST3 exhibits similarity to several sulfotransferases isolated from a wide range of animal and plant species (Weinshilboum and Otterness, 1994), thus indicating it is being a likely candidate for a PAPS binding site.

**B. Perspective for Future Works**

The availability of valuable data on F3-ST regulation, and the acquisition of two cDNA clones encoding F3-ST and a F-ST-like protein, and two cDNA encoding NDPKs would provide the basic information and tools for further studies described below.

**B.1 Intracellular localization of flavonol sulfotransferase, flavonols and sulfated flavonols at the ultrastructural level**

The flavonols and sulfated flavonols have been proposed to act as balancing components in auxin polar transports. The high concentration of both sulfated flavonols and F-ST enzyme activity in shoot tips, and their lower concentration in older tissues in this species (Hannoufa et al., 1991) are consistent with the role of a regulator that would stimulate auxin transport from the apical tissues. However, the endogenous pools and the compartmentation of those compounds have not

been demonstrated *in situ*. Moreover, flavonols and sulfated flavonols have limited taxonomic distribution (Hannoufa *et al.*, 1994). In *Flaveria spp.*, sulfated flavonols may be involved in an interactive mechanism for both the positive and negative control of auxin transport. Using immunocytochemical techniques (Ibrahim, 1990), the intracellular localization of both F-STs and their products at the ultrastructural level and in relation to their compartmentation would be necessary to evaluate this model.

**B.2 Characterization of the enzyme activity of flavonol sulfotransferase-like cDNA (pBFSTX)**

Attempts to express pBFSTX in *E. coli* and yeast systems were unsuccessful. The introduction of the pBFSTX construct under either 35S-promoter, or ubiquitin-promoter, into plant tissue by particle bombardment (Glick and Thompson, 1993) would allow the characterization of position-specific enzyme activity of pBFSTX. The presence of di-, tri-, and tetrasulfated flavonols in *F. bidentis* suggests that there are at least four F-STs corresponding to the specific substitution of 3-, 7-, 3'-, and 4'-positions of flavonols. Although different position-specific STs have been identified from the intact shoot of *F. chloraefolia* and *F. bidentis*, they account for the substitution of position 3, and 4' of flavonol to give rise to flavonol mono- and disulfates. No other cDNA clones expressing other position-specific F-ST activities have been

characterized. The characterization of the enzymatic activity of pBFSTX would, therefore, make an important contribution to the elucidation at the molecular level, of the pathway involved in polysulfated flavonol synthesis.

**B.3 Elucidation of the co-substrate, PAPS binding site and flavonol binding sites**

The high similarity between F. bidentis pNDPK-a/pNDPK-b and pBFST3 suggests that the amino acid residues 171-191 of pBFST3 might be a binding site for PAPS. However, this region is not located in the conserved region IV of the reported sulfotransferases, which consists of GXXGXXXGK and has been proposed to be related to the P-loop of a nucleotide binding site or PAPS binding site (Weinshilboum and Otterness, 1994). Photoaffinity labelling of PAPS to F3-ST protein (Otterness et al., 1991) or site-directed mutagenesis (Glick and Thompson, 1993) of the putative sites in F3-ST would, therefore, provide more evidence to confirm the PAPS binding site.

**B.4 Study of the environmental and developmental factors governing F-ST gene expression in intact plants**

In this work, F3-ST gene expression has been demonstrated in a cultured cell system to be regulated by auxin and flavonol intermediates. However, the regulation of F3-ST and F-ST-like gene expression has not been demonstrated in intact

plants. These genes might be regulated in a similar manner, or by other environmental or developmental cues. The two F-ST cDNA clones could be used as probes for this study. Moreover, the isolation of promoter regions for these two genes would allow the analysis of *cis*-acting elements regulating their expression (Glick and Thompson, 1993). In addition, the available cell suspension systems could be used for a transient or stable gene expression study, following the introduction of dissected gene constructs.

## C. Literature Cited


Bleichert E, Varin L, Hannoufa A, Ibrahim RK (1989) *In vitro* culture of *Flaveria spp.* and its potential for the biosynthesis of sulfated flavonoids. Plant Physiol **89**: S-187

Faulkner IJ, Rubery PH (1992) Flavonoids and flavonoid sulfates as probes of auxin-transport regulation in *Curcubita pepo* hypocotyl segments and vesicles. Planta **186**: 618-625

Glick BR, Thompson JE (1993) Methods in Plant Molecular Biology and Biotechnology. CRC Press, Boca Raton, Florida

Hannoufa A, Varin L, Ibrahim RK (1991) Spatial distribution of flavonoid conjugates in relation to gulcosyltransferase and sulfotransferase activities in *Flaveria bidentis*. Plant Physiol **97**: 259-263

Hannoufa A, Brown RH, Ibrahim RK (1994) Variations in flavonoid sulfate patterns in relation to photosynthesis types of five *Flaveria* species. Phytochemistry **36**: 353-356

Ibrahim RK (1990) Immunocytochemical localization of plant secondary metabolites and the enzymes involved in their biosynthesis. Phytochem Anal **1**: 49-59

Jacobs M, Rubery PH (1988) Naturally occurring auxin transport regulators. Science **241**: 346-349

Moréra S, Lascu I, Dumas C, LeBras G, Briozzo P, Véron M, Janin J (1994) Adenosine 5'-diphosphate binding and the active site of nucleoside diphosphate kinase. Biochemistry **33**: 459-467

Otterness DM, Powers SP, Miller LJ, Weinshilboum RM (1991) 3'-phosphoadenosine-5'-phosphosulfate-photoaffinity ligand for sulfotransferase enzymes. Mol Pharmacol **39**: 34-41

Varin L (1992) Flavonoid sulfation: phytochemistry, enzymology, and molecular biology. In HA Stafford, Ibrahim RK, eds, Phenolic Metabolism in Plants, Plenum Press, New York, pp 233-254

Varin L, Ibrahim RK (1992) Novel Flavonol 3-sulfotransferase: purification, kinetic properties and partial amino acid sequence. J Biol Chem 267: 1858-1863

Varin L, DeLuca V, Ibrahim RK, Brisson N (1992) Molecular characterization of two plant flavonol sulfotransferases (Flaveria chloraefolia). Proc Natl Acad Sci USA 89: 1286-1290

Weinshilboum RM, Otterness DM (1994) Sulfotransferase enzymes. In FC Kauffman, ed, Handbook of Experimental Pharmacology: Conjugation and Deconjugation Reaction in Drug Metabolism and Toxicity, Vol 112, Springer-Verlag, London, pp 45-78